Using Transgenic Plants as Bioreactors to Produce High-valued Proteins

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All experiment works reported in this thesis were performed by the author, unless stated the otherwise.

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Abstract:

Prior to the era of recombinant DNA technology, many high-valued pharmaceutical and agricultural protein products were manufactured by large-scale purification from crude extracts of animal/ plant tissues. Production lines using microbial fermentation and animal cell cultures were subsequently adopted. However, there are several disadvantages associated with these production systems, including high cost and possible contamination of animal pathogens. Recent advances in plant biotechnology enable the use of transgenic plants as bioreactors to provide an alternative platform for the production of these high-valued proteins. This research aims to test the feasibility of such system.

Two plant hosts were used for the transformation. The model plant, *Arabidopsis thaliana*, is used for quick assays, while the protein-rich *Glycine max* (soybean) is used for large scale production. To test the production scheme, two categories (pharmaceutical and agricultural) of products were examined.

Transgenic *Arabidopsis thaliana* expressing the antigenic nucleoprotein of lymphocytic choriomeningitis virus (LCMV) was generated. Successful transformation and gene expression were confirmed by Southern blot and Northern blot analyses. However, the amount of recombinant proteins produced in the transgenic lines was below the detection limit of Western blot analysis. *In vitro* translation approach, on the other hand, confirms that the recombinant DNA can indeed generate a protein product of the expected size.

For agricultural protein, the genes encoding goldfish growth hormones I and II were successfully introduced into *A. thaliana*. The presence and expression of transgenes were detected by Southern and Northern blot analyses. Crude protein extracts of these transgenic lines were subjected to radioimmunoassay. The yield was found to range from 0.0004-0.003% of total soluble proteins. *In vitro* translation using the recombinant construct was also proven to be successful.

To prepare for large-scale production, experiments were conducted to optimize the *Agrobacterium*-mediated soybean transformation system using cotyledonary nodes as explant. Parameters influencing the transformation efficiency include: (i) soybean varieties; (ii) *Agrobacterium* strains; (iii) cocultivation time for *Agrobacterium* infection; (iv) light-dark treatment of explants; (v) application of detergent, and (vi) inhibiting effects of kanamycin on plant regeneration.

摘要

在基因重組技術面世以前，許多高價值的蛋白產品都是採用由生物組織內大規模提純攝取的生產方式，及後的生產則應用於微生物或動物細胞發酵培養。但是，此等生產系統的缺點是成本高及有被動物病原體感染的危險。最新的植物生物科技利用了轉基因植物作爲生物反應器，提供另一生產系統來製造高價值蛋白。本研究主要目標是測試此生產系統之可行性。

實驗採用擬南芥菜及大豆爲轉化受體。模擬植物擬南芥菜用於快捷測試，而高蛋白作物大豆則用於大規模生產。爲了此生產模式，本研究只對兩類蛋白(醫藥及農業用)進行了測試。

淋巴細胞性脈絡叢腦膜炎病毒核蛋白抗原已被成功轉化入擬南芥菜，轉化及基因表達被南方及北方印跡分析法確定。但是，轉基因植物內重組蛋白的生產量卻低於西方印跡法的可測試水平。體外試管轉譯實驗則確定此重組基因製造出與預期大小相符的重組蛋白。

在農業用蛋白方面，金魚生長激素 I 及 II 的基因也成功轉化入擬南芥菜。導入基因的存在和表達分別被南方及北方印跡分析法確定。透過放射免疫測試，生產量爲水溶性蛋白的 0.0004 至 0.003%。此重組基因亦能成功地進行體外試管轉譯。

爲了預備大規模生產運作，本研究進行了大豆子葉節農桿菌轉化系統的最佳化測試。影響轉化效率的變項包括：（一）大豆品種；（二）農桿菌品種；（三）農桿菌感染的共培養所需時間；（四）外植體的光暗處理；（五）洗滌劑在轉化系統的應用；及（六）卡那霉素對植物再生的壓抑影響。

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General Abbreviations:

µl	Microlitre
µM	Micromolar
A. thaliana	*Arabidopsis thaliana*
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
Bp	Base pair
CAAS	Chinese Academy of Agricultural Sciences
CaMV	Cauliflower mosaic virus
Col-0	Columbia zero
cRNA	Complementary ribonucleic acid
Cys	Cysteine
DNA	Deoxyribonucleic acid
G	Gram
G. max	*Glycine max*
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
His	Histidine
Hr	Hour
Ile	Isoleucine
IU	International Unit
Kb	Kilobase pair
kDa	Kilodalton
Leu	Leucine
Lys	Lysine
M	Molar
Met	Methionine
Min	Minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
ng	Nanogram
O.D.	Optical density
PCR	Polymerase chain reaction

Phe	Phenylalanine
Pro	Proline
PVDF	Polyvinyldenefluoride
RNA	Ribonucleic acid
rpm	Revolution per minute
S	Second
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine
T-DNA	Transferred DNA
Thr	Threonine
Ti-plasmid	Tumor-inducing plasmid
Trp	Trytophan
Tyr	Tyrosine
U	Unit
UV	Ultra-viotel light
Val	Valine

Abbreviations of Chemicals:

AS	Acetosyringone
BA	Benzyl-aminopurine
BSA	Bovine serum albumin
CI	Chloroform: isoamylalcohol
$Ca(NO_3)_2$	Calcium nitrate
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro) tricyco[3.3.1.1^{3,7}] decan}-4-yl)phenyl phosphate
CTAB	Cetyldimethyethylammonium bromide
$CuCl_2$	Copper chloride
$CuSO_4$	Copper sulphate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DDT	Dithiothreitol
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
dNTPs	Deoxyribonucleoside triphosphate
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EtOH	Ethanol
FeEDTA	Ethylenediaminetetraacetic acid dipotassium salt
IBA	Indole-3-butyric acid
$K_3[Fe(CN)_6]$	Potassium hexacyanoferrate (III)
$K_4[Fe(CN)_6]$	Potassium hexacyanoferrate (II)
KCl	Potassium chloride
KNO_3	Potassium nitrate
KPO_4	Potassium phosphate
LB	Luria bertani broth, Miller
MeOH	Methanol
$MgSO_4$	Magnesium sulphate
$MnCl_2$	Manganese chloride
MOPS	3-[N-Morpholino] propanesulfonic acid
MS	Murashige & Skoog salt mixture
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate

$NaMoO_4$	Sodium molybdate
NaOH	Sodium hydroxide
NH_4OAc	Ammonium acetate
PCI	Phenol: Chloroform: isoamylalcohol
PBS	Phosphate buffer saline
PVP	Polyvenylpyrolidone
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate
TAE	Tris-acetate-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris acetate	Tris (hydroxymethyl) aminomethane acetate
Tris base	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloric acid
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucoronide

List of Figures:

Fig. 1	The schematic diagram of the full-length LCMV-NP cDNA fragment cloned in a pUC based vector	P. 72
Fig. 2	Cloning sites and important features of the V7 and W104 vectors	P. 72
Fig. 3	The gel photo showing digested and purified products of pUC-LCMV-NP and V7	P. 73
Fig. 4	Results of *Xho*l and *Xba*I digestions of the six putative V7-LCMV-NP candidates	P. 73
Fig. 5	The schematic diagram of V7-LCMV-NP	P. 75
Fig. 6	PCR screening of GV3101/ pMP90 transformants containing the plasmid W104-LCMV-NP	P. 77
Fig. 7	The schematic diagram of W104-LCMV-NP	P. 77
Fig. 8	PCR screening GV3101/pMP90 transformants containing the plasmid W104-LCMV-NP	P. 79
Fig. 9	Screening of LCMV-NP transgenic *A. thaliana*	P. 82
Fig. 10	Digestion of W104-LCMV-NP and pBluescript II KS (+) with *Xho*I and *Xba*I	P. 85
Fig. 11	PCR screening of DH5α/pBluescript II KS (+)-LCMV-NP	P. 85
Fig. 12	The schematic diagram of pKS-LCMV-NP	P. 86
Fig. 13	Southern blot analysis of LCMV-NP transgenic plants	P. 87
Fig. 14	Northern blot analysis of LCMV-NP transgenic *Arabidopsis thaliana* at heterozygous stage	P. 89
Fig. 15	Northern blot result of the four LCMV-NP transgenic *Arabidopsis thaliana* with single insert at homozygous stage	P. 89
Fig. 16	PCR amplification of full-length LCMV-NP fragment with *BamH*I and *Sac*I ends	P. 91
Fig. 17	PCR screening of DH5α/ pET-30a (+) - LCMV-NP	P. 91
Fig. 18	PCR screening of DH5α/ pKS-pET-LCMV-NP candidates	P. 92
Fig. 19	*Dra*I digestion of pET-30a (+) - LCMV-NP and pBluescript II KS (+) - LCMV-NP	P. 94
Fig. 20	*Bgl*ll digestion of the four putative pET-30a (+) - LCMV-NP candidates	P. 94
Fig. 21	PCR screening of DE3/ pET-30a (+) - LCMV-NP	P. 95
Fig. 22	Induction of LCMV-NP protein production in DE3 cells	P. 97
Fig. 23	Purification of the His-tagged LCMV-NP fusion protein	P. 97

Fig. 24	Standard curve for protein assay (for LCMV-NP recombinant protein)	P. 99
Fig. 25	Validity test of the purified anti-LCMV antibodies, the purified His-tagged LCMV-NP from DE3 cells	P. 101
Fig. 26	Western blot analysis of recombinant LCMV-NP from transgenic *Arabidopsis thaliana*	P. 102
Fig. 27	Protein dot blot analysis of recombinant LCMV-NP from transgenic *Arabidopsis thaliana*	P. 104
Fig. 28	The schematic diagram of pBluescript II SK (-) - GHI	P. 106
Fig. 29	The schematic diagram of pBluescript II SK (-) - GHII	P. 106
Fig. 30	The schematic diagram of V7-GHI	P. 107
Fig. 31	The schematic diagram of V7-GHII	P. 107
Fig. 32	Southern blot analysis of GHI and GHII transgenic *Arabidopsis thaliana*	P. 111
Fig. 33	Northern blot of homozygous growth hormones transgenic *Arabidopsis thaliana* with GHI cRNA probe	P. 113
Fig. 34	Northern blot of homozygous growth hormone II transgenic *Arabidopsis thaliana* with GHII cRNA probe	P. 113
Fig. 35	Standard curve for protein assay (for GHI and GHII recombinant protein)	P. 114
Fig. 36	Immunoassay of recombinant GHI and GHII from transgenic *A.thaliana*	P. 116
Fig. 37	Screening of pGEM-3Zf (+)-LCMV-NP candidates	P. 118
Fig. 38	The schematic diagram of pGEM-3Zf (+)-LCMV-NP	P. 119
Fig. 39	Screening of pGEM-3Zf (+)-GHI and pGEM-3Zf (+)-GHII candidates	P. 122
Fig. 40	Changing orientation of GHI cDNA insert in the pGEM-3Zf (+)-GHI clone	P. 123
Fig. 41	The schematic diagram of pGEM-3Zf(+/-)-GHI	P. 124
Fig. 42	The schematic diagram of pGEM-3Zf(+/-)-GHII	P. 124
Fig. 43	Ethidium bromide gel showing the qualities and quantities of linearized pGEM-3Zf (+)-GHI, pGEM-3Zf (+)-GHII and pGEM-3Zf (+)-LCMV-NP	P. 126
Fig. 44	*In vitro* transcription of goldfish GHI and GHII and LCMV-NP cDNA	P. 127
Fig. 45	*In vitro* translation of GHI, GHII and LCMV-NP mRNA	P. 129
Fig. 46	Shoot induction of the cotyledonary node explants in	P. 131

	differentiation medium	
Fig. 47	The shoot and root developed plantlets in root stimulating medium	P. 132
Fig. 48	Kanamycin selection of transformed soybean cotyledonary node explants	P. 134
Fig. 49	GUS staining of different parts of the explants	P. 135
Fig. 50	PCR screening of GUS gene in transformed soybean	P. 145
Fig. 51	PCR screening for the agrobacterial gene *virA*	P. 146

List of Tables:

Table 1	Milestones of scientific and technological developments which were essential to the advancement from the Green Revolution to the Gene Revolution	P. 3
Table 2	Examples of GM foods approved in U.S.A. and Canada	P. 10
Table 3	Successful examples of using transgenic plants as bioreactor to produce vaccines	P. 16
Table 4	Examples of *ex situ* applications of transgenic plants in four major categories	P. 17
Table 5	Characteristics of the seven Chinese soybean cultivars used in the experiment	P. 24
Table 6	Prevalence tests of serum antibodies against LCMV in several cities over the world.	P. 32
Table 7	Amino acid sequence identities between LASSA JOS, LASSA NIG, LCMV WE, LCMV ARM and Tacaribe and Pichinde	P. 33
Table 8	Epitopes of nucleoproteins of arenaviruses	P. 34
Table 9	Effects of exogenous growth hormone on growth promotion of fishes	P. 37
Table 10	Plant hosts, bacterial strains and vectors used	P. 39
Table 11	Commercial kits used	P. 44
Table 12	Primers and adaptors used	P. 46
Table 13	Annealing temperatures of primers used in PCR reactions	P. 63
Table 14	Chi-square test for a 3:1 green: yellow ratio of selfed progenies of successful LCMV-NP transformants	P. 81
Table 15	Raw data for the standard curve in Fig. 29	P. 98
Table 16	Concentration of crude protein extracts from LCMV-NP transgenic lines	P. 99
Table 17	Chi-square test for a 3:1 green: yellow ratio of selfed progenies of primary GHI and GHII transformants	P. 109
Table 18	Raw data for the standard curve in Fig. 35	P. 114
Table 19	Concentration of crude protein extracts from GHI and GHII transgenic lines	P. 115
Table 20	Germination frequencies of Zhe chun no. 3, Ai jiao zao and Ji lin xiao li no. 1	P. 137
Table 21	Regeneration efficiencies of Zhe chun no. 3, Ai jiao zao and Ji lin xiao li no. 1	P. 137

Table 22	Comparison of transformation efficiencies among the five Chinese soybean cultivars	P. 137
Table 23	Comparison of transformation efficiencies among Zhe chun no. 3, Ai jiao zao and Ji lin xiao li no. 1	P. 138
Table 24	Differential transformation efficiencies of Ji lin xiao li no. 1 for *Agrobacterium* strains, GV3101 and LBA4404	P. 139
Table 25	Application of vacuum infiltration during *Agrobacterium*-mediated transformation of soybean cotyledonary node	P. 140
Table 26	Effects of kanamycin	P. 141
Table 27	Effects of explant-bacterium co-cultivation duration and light/ dark treatment during germination	P. 142
Table 28	Effect of the detergent Silwet-77	P. 143
Table 29	Codon usages in *A. thaliana*, LCMV-NP, GHI and GHII	P. 154

Table of Contents

	Page
Thesis committee	i
Statement	ii
Abstract	iii
Acknowledgement	vi
General abbreviations	viii
Abbreviations of chemicals	x
List of figures	xii
List of tables	xv
Table of Contents	xvii
Chapter 1 General Introduction - Using transgenic plants as bioreactor	1
1.1 Plant as Bioreactor	1
1.1.1 Plant transformation historical milestones	1
1.1.2 Applications of transgenic plants	5
1.1.2.1Examples of *in situ* Application	5
1.1.2.2 Examples of *ex situ* application of transgenic plant	9
1.2 Plant Hosts for Transformation: *Arabidopsis thaliana* and *Glycine max*	18
1.2.1 Essential components for plant transformation	18
1.2.1.1 Marker genes	18
1.2.1.2 Promoters	18
1.2.2 *Arabidopsis thaliana*	20
1.2.2.1 *Agrobacterium*-mediated transformation	20
1.2.2.2 Transformation methods for *A. thaliana*	21
1.2.3 *Glycine max* (soybean)	22

1.2.3.1 Soybean cultivars for transformation 23

1.2.3.2 Soybean regeneration systems 24

1.2.3.3 Soybean transformation systems 26

1.3 Target Pharmaceutical and Agricultural Proteins: Lymphocytic choriomeningitis virus and Goldfish Growth hormones I and II 29

1.3.1 Production of pharmaceutical proteins 29

1.3.1.1 Lymphocytic choriomeningitis virus 30

1.3.1.2 Nucleoprotein of LCMV 33

1.3.2 Agricultural protein category 34

1.3.2.1 *Carassius auratus* 34

1.3.2.2 Growth hormones I and II 35

1.4 Hypothesis and Objectives 37

Chapter 2 Materials and Methods 38

2.1 Materials 38

2.1.1 Plants, bacterial strains and vectors 38

2.1.2 Chemicals and Regents 43

2.1.3 Commercial kits 44

2.1.4 Primers and Adaptors 45

2.1.5 Equipments and Facilities used 47

2.1.6 Buffer, solution and medium 47

2.2 Methods 48

2.2.1 Molecular Techniques 48

2.2.1.1 Bacterial cultures for recombinant DNA and plant transformation 48

2.2.1.2 Recombinant DNA techniques 48

2.2.1.3 Preparation and transformation of DH5α, DE3 and *Agrobacterium* competent cells 49

2.2.1.4 Gel electrophoresis 52

2.2.1.5 DNA, RNA and protein extractions 53

2.2.1.6 Generation of cRNA probes for Southern and Northern blot analyses 56

2.2.1.7 Southern blot analysis 56

2.2.1.8 Northern blot analysis 57

2.2.1.9 Expression of Lymphocytic choriomeningitis virus nucleoprotein (LCMV NP) in bacterial system 58

2.2.1.10 Western blot analysis for LCMV NP 59

2.2.1.11 Protein dot blot for detecting the presence of recombinant LCMV-NP generated from transgenic plants 62

2.2.1.12 PCR techniques 62

2.2.1.13 Sequencing 63

2.2.2 Plant tissue culture and transformation 64

2.2.2.1 *Arabidopsis thaliana* 64

2.2.2.2 Soybean 65

2.2.3 *In vitro* transcription and translation of target genes in rabbit reticulocyte and wheat germ systems 68

2.2.3.1 *In vitro* transcription of target genes with with Ribomix large scale RNA production systems-T7 and SP6 (Promega) 68

2.2.3.2 *In vitro* translation with rabbit reticulocyte lysate and wheat germ extract 69

Chapter 3 Results 71

3.1 Expression of Lymphocytic choriomeningitis virus nucleoprotein (LCMV NP) and goldfish growth hormones I and II (GHI and GHII) in transgenic *Arabidopsis thaliana* 71

3.1.1 Expression of LCMV-NP in transgenic *Arabidopsis thaliana* 71

3.1.1.1 Cloning of the gene encoding LCMV NP into the binary vector system W104 71

3.1.1.2 Transformation of W104-LCMV-NP into the *Agrobacterium* GV3101/ pMP90 78

3.1.1.3 Transformation of LCMV-NP cDNA into *Arabidopsis thaliana* 80

3.1.1.4 Southern blot and Northern blot analyses of transgenic plant containing the LCMV-NP cDNA 83

3.1.1.5 Production of recombinant LCMV-NP protein in DE3 cells 90

3.1.1.6 Detection of recombinant LCMV-NP protein in transgenic *A. thaliana* 98

3.1.2 Expression of goldfish growth hormones I and II (GHI and GHII) in transgenic *Arabidopsis thaliana* 105

3.1.2.1 Screening of homozygous lines of goldfish, *Carassius auratus,* growth hormones transgenic *Arabidopsis thaliana* 105

3.1.2.2 Southern blot and Northern blot analyses of transgenic plant containing the LCMV-NP cDNA 109

3.1.2.3 Detection of recombinant GHI and GHII from transgenic plant 112

3.2 *In vitro* transcription and translation of target genes in rabbit reticulocyte and wheat germ systems 117

3.2.1 Subcloning of target genes in pGEM-3Zf(+) vector 117

3.2.1.1 Subcloning of LCMV-NP fragment into pGEM-3Zf(+) vector 117

3.2.1.2 Subcloning of goldfish GHI and GHII fragments into pGEM-3Zf(+) vector 120

3.2.2 *In vitro* transcription of target genes with Ribomix large scale RNA production systems-T7 and SP6 125

3.2.3 *In vitro* translation with rabbit reticulocyte lysate and wheat germ extract systems 128

3.3 Establishment of *Glycine max* regeneration and transformation systems 130

3.3.1 The Establishment of soybean regeneration system 130

3.3.2 Establishment of soybean transformation system 133

3.3.2.1 Definition of transformation efficiency 133

3.3.2.2 Effects of plant hosts 136

3.3.2.3 Effects of *Agrobacterium* strains 138

3.3.2.4 The application of vacuum infiltration 139

3.3.2.5 Effect of kanamycin 140

3.3.2.6 Effect of cocultivation duration and light/ dark treatment during germination 141

3.3.2.7 Application of the detergent Silwet-77 142

3.3.3 Verification of transformation results by PCR screening 143

Chapter 4 Discussion 147

4.1 Expression of LCMV-NP, GHI and GHII in *A. thaliana* 148

4.2 Establishing a soybean transformation system 157

4.2.1 Plant hosts and explants 158

4.2.2 Regeneration of explants 159

4.2.3 *Agrobacterium* strains 161

4.2.4 Bacteria-plant interaction 161

4.2.5 Transient versus stable transformation 165

4.3 Conclusion and perspective 167

References 169

Appendix 186

Chapter 1 General Introduction - Using transgenic plants as bioreactor

1.1 Plant as Bioreactor

With the advent of plant genetic engineering since the 80s, technologies for isolation of valuable genes and plant transformation have improved tremendously. Integration and expression of foreign genes in plants and the feasibility of producing recombinant proteins in bulk quantities at relatively low cost have drawn much attention and interest.

Prior to the era of recombinant DNA technology, many high-valued pharmaceutical and agricultural protein products were manufactured by large-scale purification from crude extracts of animal tissues. Production lines using microbial fermentation and animal cell cultures were subsequently adopted. However, there are several disadvantages associated with these production systems, including high cost and possible contamination of animal pathogens. Recent advances in plant biotechnology enable the use of transgenic plants as bioreactors to provide an alternative platform for the production of these high-valued proteins. This research aims to test the feasibility of such system.

1.1.1 Plant transformation historical milestones

Based on the establishment of Mendel's laws of genetics in 1864, plant breeding became a science-based endeavor. Plant breeding is described as the selection of plants with desired traits after sexual exchange of genes by cross-fertilization between two parents (Goodman, *et al.*, 1987). The success in applying conventional plant breeding principles and agricultural practices to crop improvement reached its peak in the 60s, with a profound impact on agricultural production worldwide (reviewed by Kung and Wu, 1993). Increasing crop yield using such approach was subsequently referred as the "Green Revolution". Dr Noman Borlaug, the scientist initiated this technology, was awarded the Nobel Peace Prize in 1970 (Borlaug, 1983).

However, application of traditional breeding technique has encountered a major obstacle. Since breeding can occur only between two sexually compatible parents, the size of genetic pool is thus limited. To circumvent this, techniques such as protoplast fusion and somatic hybrid plants were introduced.

As plant cells are totipotent, each living cell is capable of regenerating into an entire plant identical to the one from which the cell was isolated. In the 50s, Stewart and co-workers pioneered the plant cell culture and regeneration techniques based on their discovery that plant could be developed from a single cell (Stewart, *et al.*, 1983).

Success of cell culture was extended to the production of plant protoplast by enzymatic stripping off the cell wall. In 60s, Tekebe and co-workers prepared tobacco protoplasts for efficient viral infection. Protoplasts were successfully regenerated to form intact plants with different genetic backgrounds (Tekebe, *et al.*, 1971). In 1972, Carlson and co-workers succeeded in fusing tobacco protoplasts from two genetically compatible *Nicotiana* species (Carlson, *et al.*, 1972).

Somatic hybrid plants are hybrid plants derived from the fusion of somatic cells. During the 70s, fusions between protoplasts of phylogenetically unrelated species were repeatedly attempted. Notable successful examples included the fusion produced between two sexually incompatible *Petunia* species, *P. parodi* and *P. parvifora*, and pomato, an intergeneric hybrid produced between potato and tomato (Shepard, *et al.*, 1983). However, none of these heteroplasmic fusions were able to give the desire traits for crop improvement.

Genetic transformation of plants with targeted genes was made possible via a series of scientific and technological development including DNA in 1953, plasmid in 1959, restriction enzyme and plant regeneration in the 70s, recombinant DNA techniques in 1971 and Tumour-inducing (Ti) plasmid in 1974 (Table 1). In 1983, the first transgenic petunia and tobacco were generated using plant protoplasts as the recipient cells (Horsch, *et al.*, 1984). Transformation using explants was subsequently developed to simplify the transformation procedures (reviewed by Kung and Wu, 1993).

Table 1 Milestones of scientific and technological developments which were essential to the advancement from the Green Revolution to the Gene Revolution (adopted from Kung and Wu, 1993)

Year	**Milestones**	**Pioneer scientists**
1864	Genetic laws	G. Mendel
1908-1920	Hybrid corn	G. Shull
1950	Cell culture	F. Stewart
1953	DNA	J. Watson, F. Crick
1959	Plasmid	T. Akiba, K. Ochiai
1960s	Green revolution	N. Borlaug
1970	Protoplasts & regeneration	I. Tekebe
1970	Restriction enzyme	H. Smith, D. Nathans
1972-73	Recombinant DNA	S. Cohen, P. Boyer, P. Berg
1972	Somatic hybrid	P. Carlson
1974	Ti plasmid	J. Schell
1978	Pomato	G. Melchers
1982	Direct gene transfer	
1983	Transgenic plant	

To date, the most widely adopted plant transformation techniques are mainly based on the *Agrobacterium* Ti-plasmid.

Agrobacterium tumefaciens is the causal agent of crown gall disease, and the aberrant plant growth is caused by genes (genes for octopine and nopaline catabolism and *vir* genes) present on the large plasmid (Ti plasmid) presents in the bacteria. *Agrobacterium* infection is initiated by plant wound which gives out some kind of phenolic compounds (e. g. acetosyringone) and sensed by *virA* present in the Ti plasmid. Then, signal would be passed to *virG* and further activates *vir* genes to excise a relatively small discrete portion of the plasmid known as the T-DNA (transferred DNA) from the Ti plasmid. The T-DNA includes genes encoding phytohormones such as indole-3-acetic acid (an auxin) and isopentyladenosine (a cytokinin precursor), and genes for the synthesis of opines, such as octopine and nopaline is then transferred to plant genomes (Kung and Wu, 1993).

The application of T-DNA mediated transformation lies on the observation that when the virulence region is removed, the "disarmed" T-DNA retains its ability to integrate into the plant genome, if the functions of the *vir* genes are provided *in trans*. The key elements on the T-DNA for successful integration are the 25 bp imperfect direct repeats present as the left and right boundaries, which delimit the region of T-DNA. Target genes inserted into the T-DNA fragment between the left and right T-DNA borders will be simultaneously transferred into the plant genome (Kung and Wu, 1992).

Two major classes of Ti-plasmid vector systems commonly employed are binary and cointegrate vectors, whereas the *vir* genes functions (essential for the DNA transfer process) are provided by a helper Ti-plasmid or on the same Ti-plasmid, respectively (Stachel, *et al.*, 1985).

In cointegrate vector systems, the target genes are first cloned in a cloning vector in which the cloning site is flanked by DNA sequences homologous to the T-DNA. When the construct is transferred into an *Agrobacterium*, homologous recombination occurs at the T-DNA region and the cloning vector will be integrate into the resident Ti-plasmid in the *Agrobacterium* host. Depending on the *vir* genes functions of the Ti-plasmid, the T-DNA containing the target genes will be integrated into the plant genome.

In binary vector systems, the target genes are cloned into the cloning sites flanked by the left and right T-DNA borders. The *vir* gene functions are provided *in trans* by a helper plasmid in which the original T-DNA was removed or defective in gene transfer (Zambryski, *et al.*, 1983).

In case where *Agrobacterium*-mediated transformation is not effective (e.g. in most monocotyledonous plants), particle gun bombardment techniques will be employed. Other methods such as the introduction of DNA into plant protoplasts via electroporation, polyethylene glycol (PEG) mediated gene uptake, co-precipitation with calcium phosphate or particle bombardment facilitates the transformation of monocotyledonous plants, which are in general not readily susceptible with

Agrobacterium infection (Christou, 1996; Hansen and Wright, 1996; Leighton, 1999).

1.1.2 Applications of transgenic plants

The potential applications of the transgenic plant techniques are enormous and can be roughly classified into two categories. Molecular farming focuses on the utilization of plants or cultured plant cells as bioreactors to produce target proteins for subsequent purification and utilization (*ex situ* application), while molecular breeding exploits transgenic protein expression that introduces desirable traits (such as resistance to herbicides, insects or pathogen attacks) into target plants (*in situ* application) (Franken, et al., 1997; Goddijn and Pen, 1995).

1.1.2.1 Examples of *in situ* application

Improvement of crop nutrition

One main target of genetic engineering of crop plants is to improve the amino acid composition of seed proteins. For instance, lysine is limiting in cereal grains, whereas sulphur-containing amino acids are limited in legume seeds. These imbalances of essential amino acids could lead to detrimental consequences for people adopting vegetarian diet of limited diversity (reviewed by Tabe and Higgins, 1998).

Advances in plant tissue culture and gene transfer technologies enable the modification of amino acid content in plants. One approach is to manipulate the regulation of amino acid biosynthesis in order to increase the content of methionine in legume. Both lysine and methionine are members of aspartate family amino acids, and aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS) are key enzymes involved in the biosynthetic pathway. Genes encoding feedback insensitive forms of AK and DHPS were fused downstream to a seed-specific promoter and transferred to plants. This approach resulted in a 100-fold increase in free lysine contents in transgenic soybean and canola (Falco, *et al.*, 1995). Seed-specific expression of a feedback insensitive AK alone resulted in a 17-fold increase in free threonine and 3-fold increase in free methionine in transgenic tobacco (Falco, *et al.*, 1995).

Another approach is to insert and express foreign genes encoding sulphur-rich or lysine-rich proteins in transgenic plants. Attenbach and co-workers reported that when a chimeric gene encoding the Brazil nut methionine-rich 2S albumin protein (BNA) under the control of a seed-specific, developmentally regulated promoter from *Phaseolus vularis* phaseolin gene was transferred to tobacco, the BNA protein accumulated to approximately 8% of the total extractable proteins and resulting in 30% increase of seed methionine (Atlenbach, *et al.*, 1989). Similar approach to improve the seed lysine contents by introducing the winged bean lysine-rich protein (LRP) was also attempted (Cheng, 1999).

Besides balancing the amino acid composition, essential elements such as vitamins were also engineered into crop. Predominant rice (lacking vitamin A) consumption resulting in vitamin A deficiency affecting 26 countries in Asia, Africa and Latin America. The World Health Organisation estimates that 2.8 million children under five currently have severe vitamin-A deficiency, known as xerophthalmia, that causes permanent blindness in 500,000 children a year. Recombinant DNA technology was thus used to introduce genes encoding the entire β-carotene biosynthetic pathway into rice and the resulting transformed rice provides high quality of β-carotene in the endosperm of rice (Ye, *et al.*, 2000).

Vitamin E content of plants was also elevated by metabolic engineering. Daily intake of vitamin E in range of 100 to 1000 IU is associated with decreased risk of cardio vascular disease and some cancers, improved immune function and slowing of the progression of a number of degenerative human condition. However, these therapeutic levels of vitamin E from average diet are nearly impossible. Thus, substantial increase in the α-tocopherol (vitamin E) content of major crops are needed to provide the public with dietary sources of vitamin E that can approach the desired therapeutic levels. As the limiting factor of α-tocopherol production is the limited amount of γ-tocopherol methyltransferase (γ-TMT). Stintani and DellaPenna constructed an *A. thaliana* γ-TMT overexpresser. The transgene was driven by the seed-specific carrot DC3 promoter. HPLC analysis showed that seed-specific overexpression of *A. thaliana* γ-TMT gene increased seed α-tocopherol level more than 80-fold (Shintani and DellaPenna, 1998).

Improvement of crop yield

Another major aspect of crop improvement is to increase yield. Many agronomically important crop species, including rice and wheat, assimilate carbon via the C_3 photosynthetic pathway. The pathway suffers from oxygen inhibition due to the oxygenase reaction of ribulose 1,5-bisphosphate carboxylase/ oxygenase (Rubisco) and the subsequent loss of the already assimilated carbon (as carbon dioxide) and nitrogen (as ammonia) via photorespiration. By contrast, in the C_4 pathway, atmospheric carbon dioxide is initially fixed onto oxaloacetate, a C_4 acid in the mesophyll cytosol, by phosphoenolpyruvate carboxylase (PEPC). The PEPC possesses high affinity to the substrate bicarbonate and is not inhibited by oxygen. Thus, C_4 plants exhibit high photosynthetic capacity as well as high water and nutrient utilization efficiencies. Ku and co-workers introduced the intact gene of maize PEPC to the C_3 crop rice by *Agrobacterium*-mediated transformation (Ku, *et al.*, 1998). The transgene was placed under the control of the PEPC promoter. The activities of the PEPC produced in leaves of some transgenic rice were two- to three-fold higher than those in maize and accounted for up to 12% of the total leaf soluble protein in transgenic rice. Besides, the efficiency of photosynthesis in those transgenic rices was reported to increase for more than 30% (Ku, *et al.*, 1997).

Resistance to pathogens

Pathogen resistances are agronomically important traits of crop plants. It can be achieved by providing naturally occurring resistance-mediated or synthetic resistant proteins by transformation of the corresponding genes into the target plants.

For example, gene encoding Trichosanthin, a ribosome-inactivating protein (RIP) from the Chinese medicinal herb, *Trichosanthes kirilowii*, was transferred to tobacco. The product of RIP accounted for up to 0.1% of the total soluble protein in the transgenic plants that were found to be completely resistant to mechanical inoculation of turnip mosaic virus (Lam, *et al.*, 1996).

On the other hand, resistance against fungal pathogen, could be achieved by constitutively expressing an enzyme (e.g. chitinase, β-1,3-glucanase) hydrolyzing

fungal cell wall (Lin, *et al.*, 1995).

For insect resistant traits, it was reported that transgenic rice expressing the enzyme inhibitor acquired resistance to yellow stem borer and striped stem borer. Snowdrop lectin in transgenic potato also showed significant anti-feedant effect on the tomato moth (For review, see Franken, et al., 1997).

Since the late 80s, the insecticidal crystal toxin genes from *Bacillis thuringiensis* have been used to confer insect resistant trait in transgenic plants. Recent studies utilizing modified synthetic crystal toxin genes as an effective pest control in canola, soybean, rice, alfafa and tobacco were underway (for review, see Franken, *et al.*, 1997).

Tolerance to abiotic stresses

Abiotic stresses including salinity, heat, cold and drought limit the arable across worldwide. Therefore, construction of transgenic plants tolerant to these stresses is of utmost significance. For example, a gene from *E. coli* encoding mannitol 1-phosphate dehydrogenase was transferred into tobacco and expressed constitutively. This enzyme catalyzes the reversible interconversion of fructose-6-phosphate and mannitol-1-phosphate. The transgenic plants accumulated mannitol in leaves and roots and gained tolerance to high-salinity stress. This supported that plants accumulating osmolytes can withstand drought and high salinity (Kahl and Winter, 1995).

Slow ripening fruits

In order to reduce the loss caused by spoilage of fruits and vegetables during transportation, anti-sense technology was employed to reduce/ eliminate the functions of genes responsible for ripening. Oeller and co-worker engineered tomatoes expressing an anti-sense mRNA of ACC (1-aminocyclopropane-1-carboxylic acid) synthase. ACC synthase was enzyme essential for the production of ethylene which is a key plant hormone for the ripening process. The inhibition of ripening can then be released by exogenous application of ethylene after transporting to the destination

desired (Oeller, *et al.*, 1991).

Tolerance to herbicide

Weeds reduce crop yield by competing for nutrients and reducing available solar energy to crop plants. To remove weeds, herbicides are usually added before planting of crops. However, herbicides cannot be applied to field containing crop plants unless these crop plants are resistant to herbicides. Roundup is a major herbicide and its active ingredient is glyphosate that will inhibit the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme for the synthesis of aromatic amino acids. Gene encoding a glyphosate insensitve EPSPS was introduced into crops, such as soybean. The application of Roundup will then kill weeds, but not the transgenic crops (named as "Roundup-Ready") (Padgette, *et al.*, 1991).

Examples of genetically modified food in market

To date, many transgenic crops have been produced and some are approved to sell in market. Table 2 summarized some examples of them.

1.1.2.2 Examples of *ex situ* application of transgenic plants

Production of carbohydrates

The quality of starch is determined by the relative ratio of amylopectin (branch-devired starch) and amylose (straight-chained starch). Transgenic potato with foreign gene encoding the granule-bound starch synthase (GBSS) in the anti-sense orientation produced much less amylose. This “amylose-free” potato starch can be used in preparation of microwave-ready food, as it gives clear pastes that do not deteriorate (reviewed by Goddijn and Pen, 1995).

Besides, the amount of starch in plant storage organs could be increased by inserting a mutated bacterial gene (*glgC16*) encoding the ADP glucose pyrophosphorylase. Low-starch potato lines transformed with this gene produced

tubers with around 60% more starch than the untransformed wild type (Shewmaker, *et al.*, 1994).

Table 2 Examples of GM foods approved in U.S.A. and Canada. Data adopted from the following websites: http://www.hc-sc.gc.ca/english/food.htm#novel and www.cfsan.fda.gov/.

Modified traits	**Crop plants**
Herbicide resistance/ tolerance	Canola (Oilseed Rape), Corn, Cotton, Flax, Soybean, Sugar beet, Wheat
Insect resistance	Corn, Cotton, Potato, Tomato
Virus resistance	Papaya, Potato, Squash
Delayed ripening	Cantaloupe, Tomato
Male sterility	Canola, Radicchio rosso
Oil contents	Canola, Soybean
Phosphate contents	Canola

Production of fatty acids

In searching for an alternative saturated fatty acid source to cocoa butter, stearic acid in canola was increased by transforming with an anti-sense stearoyl-ACP desaturase cDNA under the control of a napin or ACP promoter. The activity and amount of stearoyl-ACP desaturase were greatly decreased, resulting a marked increase in stearic acid (< 2 % to 40 %) (Knutzon, *et al.*, 1992).

Production of biodegradable polymer

In order to produce environmental friendly biodegradable polymers, *Alcaligenes eutrophus* genes encoding beta-ketothiolase, aceto-acetyl-CoA reductase and polyhydroxyalkanoic acid (PHA) synthase, which catalyse the formation of polyhyhroxylbutyrate polyesters, from *Alcaligenes eutrophus* were transformed into *A.thaliana*. Synthesis of these enzymes in cytoplasm led to the accumulation of 100μg poly-beta-hydroxy butyrate (PHB) per gram fresh weight. Redirecting the PHB biosynthetic pathway from cytoplasm to plastid resulted in a 100-fold increase in the final PHB production (Poirier, *et al.*, 1992).

Production of industrial enzymes

(i) α-amylase

α-amylases are widely used in industry for the hydrolysis of α-1,4-glycosidic linkages in the starch components amylose and amylopectin. The reaction is important in industrial processes such as starch processing and alcohol liquefaction, baking (for increasing bread volume), juice and wine clarification and detergent industry.

To produce α-amylase in plants, chimeric α-amylase genes fused with amylase signal peptide from *Bacillus licheniformis* or from tobacco pathogenesis-related protein S (PR-S protein) and transcribed under the control of CaMV 35S promoter were transformed into tobacco. The enzyme accumulated up to 0.5% of the soluble protein in transgenic tobacco plants and both signal peptides

showed equal ability in secreting of α -amylase to extracellular space. The thermostable (95-100°C) recombinant α-amylase synthesized from plants exhibited similar biological activities in starch degradation when compared with those generated in microbial fermentation (Pen, *et al.*, 1992).

(ii) Xylanases

Xylan hydrolysis is essential in paper industry, improvement of nutritional value of animal feed, clarification of must and juices, liquefaction of vegetables and fruit, and production of bread. Xylanolytic enzymes include acetylesterase, α -L-arabinofuranosidase, α-glucuronidase, xylanase and β-xylosidase.

Two xylanases encoding genes, *xynZ* and *xynD*, isolated form the thermostable sewage bacterium *Clostridium thermocellum* and ruminal bacterium *Ruminococcus flavefaciens* respectively, were used to transform tobacco. The two xylanases encoding genes transcribe under the control of CaMV 35S promoter were preloaded with a proteinase inhibitor signal peptide to target the respective proteins into apoplastic space. Both transgenic plants generated active xylanases which were expressed in the apoplast and exhibited stabilities in acidic media and heat conditions (Owen and Pen, 1996).

(iii) Phytases

Phytic acid is the main storage form of phosphorus in many seeds. However, when used as feed, phytate phosphorus is not normally digestible by animal. In order to optimize phosphorus utilization by converting of phytate into myoinositol and inorganic phosphate, enzyme phytase from *Asperigillus niger* was fused with a tobacco PR-S signal peptide under the control of a modified CaMV 35S promoter with duplicated enhancer and a leader sequence from alfalfa mosaic virus (AMV) RNA4. The construct was transformed into tobacco by *Agrobacterium*-mediated transformation. When the transformants were grown for 3 weeks after transfer to soil, the highest phytase expression level was estimated to be 1.7% of the total soluble protein in leaves and 1% of the total soluble protein in seeds. Due to the presence of

the signal peptide, the recombinant phytase was secreted to the extracellular space and the extracellular fluid isolated exhibited specific phytase activity (Verwoerd, *et al.*, 1995).

In transformants grown for seven weeks after transferred to soil, the phytase levels increased approximately 20-fold, up to a maximum level of 14.4% of total soluble protein. Since there was no significant increase in the mRNA, the regulation of phytase amount is likely at the protein level. The high stability of transgene product in the extracellular space of leaves may account for such high levels of non-plant protein produced in transgenic plants (Verwoerd, *et al.*, 1995).

In addition, *A. niger* phytase was also transformed into canola (*Brassica napus* cv Westar) under the control of a seed-specific cruciferin A promoter and the cruciferin signal peptide. The highest phytase level obtained was estimated to be 9.3% of total soluble proteins in seeds. (Owen and Pen, 1996).

Production of pharmaceutical compounds

In addition to the production of industrial enzymes in transgenic plants, biosynthesis of human proteins for pharmaceutical uses was also attempted. Using transgenic plants as bioreactor to produce complex therapeutic proteins gives several advantages, including the ease of genetic manipulation, lack of potential animal pathogen contaminations, low cost of large-scale production, and partial conservation (between animals and plants) of protein modification machinery.

(i) Human protein C

Human protein C (hPC), a highly processed serum protease of the coagulation/ anticoagulation cascade, was an example of human enzymes produced in transgenic plants. The hPC proteins were produced at low levels in transgenic tobacco leaves (Cramer, *et al.*, 1996). Besides, CropTech researchers also produced the human lysosomal enzyme glucocerebrosidase (hGC) in transgenic tobacco. This glycoprotein was proven to be highly effective in reducing disease manifestations in patients with Gaucher's disease. The hGC cDNA was placed under the control of an inducible plant

promoter in transgenic tobacco. The plant-derived hGC was glycosylated and enzymatically active. The hGC production was estimated to be greater than 1 mg/g fresh weight of leaf tissue crude extracts (Cramer, *et al.*, 1996).

(ii) Antibodies

As mentioned above, pharmaceutical proteins including antibodies were conventionally produced by microbial fermentation. However, production of recombinant immunoglobulins in *E. coli* can be hindered by low yield and low solubility (folding pathway of recombinant proteins is often impaired in prokaryotic cells). To circumvent this, several systems have been established to express complete antibodies in transgenic plant cells (Conrad and Fiedler, 1998).

The initial strategy to produce antibody in plants was to express each immunoglobulin chain separately in different plants and followed by cross-pollination of the individual heavy- and light-chain-expressing plants. Most groups used the constitutive CaMV 35S promoter to drive gene expression. The yield of recombinant antibody is consistently high - between 1 and 5% of total plant protein (Hiatt, *et al.*, 1989). First successful story was reported by Hiatt and co-workers in 1989 (Hiatt, *et al.*, 1989). The cDNAs encoding gamma and kappa immunoglobulin chains derived from a mouse hybridoma mRNA were used to transform tobacco explants. After regeneration, plants expressing gamma or kappa antibodies were crossed. Functional antibodies accumulated up to 1.3% of total leaf proteins. Specific antigen binding activities of plant-derived antibodies were similar to the hybridoma-derived counterparts (Hiatt, *et al.*, 1989). Subsequently, Ma and co-workers generated four transgenic *Nicotiana tabacum* plants expressing the murine monoclonal antibody K chain, and the hybrid IgA-G antibody heavy chain against *Streptococcus mutans*. The yield of antibody was between 10-80mg/kg fresh weight of plant materials. The human trial report demonstrated that the plant SIgA/G was as protective and specific as the native mucosal antibody over a period of four months (Ma, *et al.*, 1995; 1998).

Successful results were also obtained by using double-transformation techniques to introduce both the heavy- and light-chain genes into the same plant cells simultaneously. The yield of antibody was estimated to be 0.055% of total soluble

protein. Alternatively, both the light- and heavy-chain genes were placed on a single T-DNA. In this case, the promoter and terminator were carefully chosen to coordinate expression of the two transgenes. Expression levels up to 1.1% of total soluble proteins were reported (reviewed by Ma and Hein, 1995).

(iii) Vaccines

Conventionally, subunit vaccines were based upon recombinant cell-culture expression systems. However, for commercial-scale production, these systems require fermentation technology and stringent purification protocol to produce sufficient amount of recombinant protein for oral delivery. This fermentation-based subunit vaccines production is a prohibitively expensive technology for developing countries where novel oral vaccines are urgently needed (Mason and Arntzen, 1995). In the early 90s, Arntzen and co-workers started to make plant-based vaccines, including the edible vaccines (Mason, *et al.*, 1996). The idea behind the edible vaccines is to reduce administrative cost of vaccine transport, storage and injection that may add up to more than 88% of the total cost of a vaccine (Derek and Hagan, 1994).

Antigens that have been successfully produced in transgenic plants included hepatitis B surface antigen, subunit B of the heat labile toxin (LT) of enterotoxigenic *E. coli* (causing diarrhea), subunit B of cholera toxin (CT), capsid protein of Norwalk virus (causal agent of acute gastroenteritis), malarial epitope from *plasmodium spp.*, V3 loop of HIV-1 and rabies viral Drg24 antigen (Table 3).

Table 4 summarized several *ex situ* examples in the four major product categories, including carbohydrates, fatty acids, pharmaceutical protein and industrial enzymes produced by transgenic plants.

Table 3 Successful examples of using transgenic plants as bioreactors to produce vaccines.

Antigen	**Gene donar**	**Plant recipient**	**Promoter**	**Remarks**	**Yield**	**Immuno-response test[a]**	**References**
CT-B	*Vibrio cholerae*	Potato	mannopine synthase P2 promoter	CTB leader sequence at 5' end and ER retention signal (SEKDEL) at 3' end	0.3% of soluble protein	Positive	Arakawa, *et al.*, 1997 & 1998
LT-B	ETEC	Tobacco/ potato	CaMV 35S	TEV 5'-UTR and vsp B 3' flank	Tobacco: 14μg/ g; Potato: 110μg/ g	Positive	Haq, *et al.*, 1995
Capsid protein	Norwalk virus	Tobacco/ potato	CaMV 35S	TEV 5'-UTR at 5' end	Tobacco: 0.23%; Potato: 10-20μg/ g of tuber protein	Positive	Mason, *et al.*, 1996
Surface antigen	Hepatitis B virus	Tobacco	CaMV 35S	Duplicated enhancer linked to the tobacco etch virus 5' non-translational leader sequence	0.01% of soluble protein	Positive	Thanavala, *et al.*, 1995
Malaria epitope	*Plasmodium spp.*	Tobacco	ND	Expressed on the surface of recombinant tobacco mosaic virus	0.3% of virion weight	Positive	Turpen, *et al.*, 1995
V3 loop of HIV-1	HIV	Tobacco	ND	Recombinant tobacco mosaic virus with VB loop of HIV-1 gene was inoculated	ND[b]	Positive	Yusibov, *et al.*, 1997
Drg 24 antigen	*Rabies* virus	Tobacco/ potato	ND	Recombinant tobacco mosaic virus with *in vito* transcripted RNA products were inoculated	ND[b]	Positive	Yusibov, *et al.*, 1997 & Modelska, *et al.*, 1998

[a] not determined

[b] positive: antibody raised by the recombinant antigens

Table 4 Examples of *ex situ* applications of transgenic plants in four major categories.

Compounds	**Gene donors**	**Applications**	**Plant hosts**	**References**
Carbohydrates				
Cyclodextrins	*Klebsiella pneumoniae*	Food, pharmaceutical	Potato Tobacco,	Oakes, *et al.*, 1991; Sevenier, *et al.*, 1998
Fructans	*Bacillus subtilis*	Industrial , food	sugar beet, tobacco	Ebskamp, *et al.*, 1994
Lipids				
Polyhydroxy-butyric acid	*Alcaligenes eutrophus*	Biodegradable plastics	*Arabidopsis*, oilseed rape, soybean	Poirer, *et al.*, 1995
Saturated fatty acid	*Brassica rapa*	Food, confectioneries	Oilseed rape	Knutzon, *et al.*, 1992
Pharmaceutical polypeptides				
Antigens	Bacteria, viruses	Vaccines	Tobacco, potato	Haq, *et al.*, 1995; Mason, *et al.*, 1992;
Growth hormone	Trout	Growth stimulation	Tobacco, Arabidopsis	1995 Bosch, *et al.*, 1994
Industrial enzymes				
Xylanase	*Clostridium thermocellum*	Liquefaction of starch	Tobacco	Herbers, *et al.*, 1995
Alpha-amylase	*Bacillus licheniformis*	Animal feed, paper and pulp, baking	Tobacco, alfalfa	Pen, *et al.*, 1992; Austin, *et al.*, 1995

1.2 Plant Hosts for Transformation: *Arabidopsis thaliana* and *Glycine max*

1.2.1 Essential components of plant transformation

1.2.1.1 Marker genes

Markers play a crucial role to identify and select for successful transformation events. Selectable marker confers a dominant phenotype on transformed cells because they invariably result in the addition of a new trait. They may allow living cells, tissues, or whole plants to grow under conditions that prevent the growth of untransformed tissues. For example, antibiotic resistance gene derived from the bacterial transposon *Tn5* encoding the neomycin phosphotransferase II is a selectable marker conferring resistance to the aminoglycoside antibiotics. Another type of markers show negligible effect on cell survival, but give transformed cells distinguishable physical characteristics. The β-D-glucuronidase (GUS) reporter gene is typical example (Kung and Wu, 1993).

1.2.1.2 Promoters

In order to produce a functional level of target proteins in transgenic plants, gene encoding the protein of interest must be transcribed at an appropriate level. Effective transcription can be achieved by using suitable promoters. Promoters currently used in transgenic plants are normally derived from the T-DNA of Ti-plasmids and from plant pathogenic viruses. Their popularity can be attributed to their ability to function in most plant tissues, their strength and their broad species compatibility (Owen and Pen, 1996).

T-DNA promoters

The opine biosynthesis genes *nos* (nopaline synthase), *ocs* (octopine synthase)

and *mas* (mannopine synthase) provided the promoters for the pioneer works of plant genetic engineering and are still widely used for driving selectable marker genes. *Nos*, *ocs* and *mas* promoters are compact and of less than 400bp each. The expression of these promoters is often assumed to be constitutive, although they are clearly under developmental control. The *nos* promoter is highest in the older leaves, stem and flowers. The *ocs* promoter is best expressed in younger leaves, meristematic regions of shoot and roots. The *mas* promoter is best expressed in roots and older leaves (Guevara-Garcia, *et al.*, 1998; Ni, *et al.*, 1995).

Viral promoters

The double-stranded caulimovirus family has provided the most widely used promoter for transgene expression in plants: the cauliflower mosaic virus (CaMV) 35S promoter. The CaMV 35S promoter is generally stronger than the T-DNA promoters and is expressed constitutively and ectopically in most tissues of most plants. Correct initiation of transcription from the 35S promoter is dependent on proximal sequences that include a TATA element, whereas the rate of transcription is determined by sequences that are dispersed over 300bp of the upstream DNA (Kay, *et al.*, 1987; Gatz, *et al.*, 1991).

Other promoters

Besides T-DNA promoters and the CaMV 35S promoter, there are also other promoters that express constitutively, including actin and ubiquitin promoters. The actin promoter has been used in wheat and rice transformation. The ubiquitin promoter has been successfully employed in monocotyledonous plants to achieve strong expression of marker genes. Distinguished from constitutive promoters, tissue-specific promoters are particularly useful to produce proteins that must be concentrated in certain organs to limit toxicity or to improve harvesting efficiency. Patatin, *BET1* and phaseolin promoters that express specifically in tubers, endosperms and seeds, respectively, are examples in current use (Grierson, *et al.*, 1994; Hueros, *et al.*, 1995; Altenbach, *et al.*, 1989).

1.2.2 *Arabidopsis thaliana*

In this project, *Arabidopsis thaliana* was used as a quick assessment system to test the efficiency of expression of the target genes in transgenic plants.

Arabidopsis thaliana, originally an inconspicuous flowering weed, has long been used as a model plant for experiment in genetics because of its small physical size, large seed set and relative ease of large-scale mutagenesis. In addition, its generation time is relatively short (about five to six weeks) and is able to grow well in laboratory setting. These features allow easy and inexpensive indoor maintenance of large populations of *A. thaliana*.

Furthermore, *A. thaliana* is also popularly used as a subject to tackle problems in plant physiology, biochemistry and development via molecular genetics approaches. It is due to the small size of its nuclear genome (haploid nuclear genome is approximately 70, 000 kb) that allows rapid and repetitive screening of gene libraries. For instance, cosmid libraries representing several genomes worth of *A. thaliana* DNA can be plated on a single large Petri dish. Besides, the haploid genome consists of five chromosome and numerous mutants were available. Thus, it is easy to induce new mutations, screen or select for desire phenotypes and determine the locations of the genes revealed by mutant phenotypes. Recently, gene chips of *A. thaliana* are also developed. It greatly facilitates systematic isolation of differentially expressed genes. Function of target genes can be further studied by construction of overexpressers and underexpressors using well-established transformations (Meyerowitz, 1989).

1.2.2.1 *Agrobacterium*-mediated transformation

As mentioned in Section 1.1.1, the *Agrobacterium* provides an effective transformation system because of the relative low operation and affordable costs. Besides, transgenic plants obtained often contain a single copy insert, preventing expression and recombination problems associated with high copy number of the

transgene.

1.2.2.2 Transformation methods for *A. thaliana*

Agrobacterium-mediated leaf-disc transformation

The status of plant host used for transformation is one of the major determining factors. Horsch and co-workers first demonstrated that *Agrobacterium* could effectively transform leaf-disk explants of *A. thaliana* (Horsch, *et al.*, 1985). Surface-sterilized leaf disks were co-cultivated with *Agrobacterium* containing the target gene and kanamycin resistant marker gene in a binary vector system for 2 days. The leaf disks were subsequently cultivated on selective medium and shoots were regenerated within 2-4 weeks.

***Agrobacterium* vacuum infiltration**

Although leaf-disk transformation could yield transgenic *A. thaliana*, this transformation method is time consuming and requires skilled labor and relatively expensive laboratory facilities. By contrast, *Agrobacterium* vacuum infiltration offers a simple alternative. This is accomplished by *in planta Agrobacterium*-mediated gene transfer by vacuum infiltration of adult *A. thaliana* plants, as first reported by Bechtold in 1993 (Bechtold, *et al.*, 1993). In this method, *A.thaliana* is first grown to flowering stage before application of *Agrobacterium* to the whole plant via vacuum infiltration in a sucrose-hormone growth medium. The treated plants are allowed to shed seeds and successful transformation events are screened on selective medium using selective markers present on the T-DNA (Chang, *et al.*, 1994).

Using this technique, most transformed progenies are genetically identical and thus minimize the somaclonal variation during plant tissue culture and regeneration steps. Primary transformants are typically heterozygous for the transgene, suggesting that transformation may occur after the divergence of anther and ovary cell lineages. Likely targets are the gametophyte-progenitor tissues, mature

gametophytes or recently fertilized embryos (Chang, *et al.*, 1994).

Agrobacterium floral dip transformation

Recently, Clough and Bent reported that transformation of *A. thaliana* could be obtained at high rate (0.5-3%) simply by dipping flowering plants in *Agrobacterium* that are suspended in a solution containing sucrose and the surfactant Silwet-77. Silwet-77 is a low phytotoxicity surfactant that will reduce surface tension and thus enhance the entry of bacterium into the apparently inaccessible plant tissues (Cloug and Bent, 1998).

1.2.3 *Glycine max* (soybean)

Glycine max (soybean) is economically the most important legume in the world, providing protein for millions of people and raw materials for many chemical industries. It is believed that soybean originated in China, probably in the north and central regions, 4000-5000 years ago. Soybean was successfully introduced to Europe and North America in 18^{th} and 19^{th} century, respectively. To date, U.S.A. has become the largest soybean exporting country. The net exporting value in 1998 was US$ 4 884 515 000 (Data from http://www.fao.org).

The chemical composition of soybean generally includes oil (~20% by dry weight), protein (~40% by dry weight), sugar, phosphorus, potassium, calcium and vitamins. For proteins, its amino acid composition is closer to the animal protein counterpart than most other crops. The oils include large amount of phosphorus and the phosphatide content of soybean flour is about 2 % and is a mixture of lecithin and cephalin (Liu, 1997).

In this research, the high seed protein plant soybean was chosen as one of the plant host for establishing bioreactor to produce high-valued proteins. However, regeneration and transformation technologies for soybean are not as mature as those in the model plants such as *A. thaliana* and tobacco. Successful soybean

transformation and generation of stable transgenic soybean lines were not routine process and were only reported in a few cases (mainly in seed companies). Therefore, optimization of soybean regeneration and transformation platform was chosen as one of my project goals.

1.2.3.1 Soybean cultivars for transformation

In this research, seven Chinese soybean cultivars were used as the plant hosts for transformation. They included Ji lin xiao li no. 1, Ji lin no. 30, Ji lin no. 36, Hei long no. 37, Chang nong no. 5, Zhe chun no. 3 and Ai jiao zao. The first five cultivars were used as experimental materials for soybean transformation in China basing on their high regeneration and differentiation efficiencies (王連錚，王金陵，１９９２). Besides, they were also ideal for actual production because of their high yields and protein contents. However, they are cultivars adapted to grow in Northern regions of China and perform poorly in Southern region such as Hong Kong. The later two cultivars, Zhe chun no. 3 and Ai jiao zao, were subsequently employed, as they can grow well in the South and are also common experimental materials for soybean researches (Table 5).

Table 5 Characteristics of the seven Chinese soybean cultivars used in the transformation experiment. (王連錚，王金陵．１９９２)

Soybean cultivar	Protein content (%)	Fat content (%)	Callus formation efficiency (%)	Cotyledonary node explant differentiation efficiency (%)	Other characteristics
Ji lin xiao li no. 1	44.9	16.1	50	90	
Ji lin no. 36	N.D.[a]	N.D.[a]	40	10	Insect resistant
Hei long no. 37	38	21.6%	70.8	N.D.[a]	
Ji lin no. 30	N.D.[a]	N.D.[a]	64	40	tolerant to water-logged soil
Zhang long no. 5	40.2	19.9	N.D.[a]	50	
Zhe chun no. 3	48	18	N.D.[a]	N.D.[a]	tolerant to water-logged soil and resistant to mosaic disease
Ai jiao zao	37	16.3	N.D.[a]	69	high yield

[a]N.D.: Not determined

1.2.3.2 Soybean regeneration system

For a successful transformation event, plant cells involved should be both regeneration-competent and transformation-competent.

It is the totipotency of plant cells that underlies most plant regeneration systems. Soybean can regenerate via two distinct paths - somatic embryogenesis and shoot morphogenesis, which are both controlled by plant hormones and other factors

present in the culture medium. Skoog and Miller demonstrated that organogenesis in *in vitro* cultures was controlled by the ratio of exogenous auxins and cytokinins concentrations (Skoog and Miller, 1957). Thus, the ratio of auxins and cytokinins concentration must be optimized for each plant species to stimulate shoot and root regeneration from the explants. Media with higher levels of auxins relative to cytokinins lead to induce root regeneration. Conversely, media with lower levels of auxin relative to cytokinin lead to induce shoots. Shoot morphogenesis is the process of shoot meristem organization and development. Shoots grow out from a source tissue will be excised and rooted to obtain intact plants.

Somatic embryogenesis in soybean was first reported by Christianson and co-workers in 1983. During somatic embryogenesis, an embryo containing both shoot and root axes is formed from somatic plant tissues (tissue other than germ-line). Such somatic embryos were developed from either microspores or somatic tissues (Christianson, *et al.*, 1983). In 1988, Finer described a somatic embryogenesis system in which embryos were induced from immature soybean cotyledons by placing the explant on high level of 2,4-dichlorophenoxyacetic acid (2,4-D, an auxin). Histological analysis of proliferating embryos indicated that new somatic embryos were initiated at or near the surface origin of new embryos and this tissue is thus a suitable target for transformation (Samoylov, *et al.*, 1998).

Shoot morphogenesis was first reported by Wright and co-workers in 1986 (Wright, *et al.*, 1986). When cotyledonary node explants placed on medium containing 6-benzylaminopurine (BAP, a cytokinin), shoots formed *de novo* from subepidermal tissues. This system was subsequently employed for transformation. The subepidermal, multicellular origin of the shoots are recognized as the target cells for transformation. This transformation system gives rise to new shoots within the meristematic tissue and lessen problems associated with chimerism. This method allows proliferating shoots to form rooted plants in less than 3 months, while plant recovery from somatic embryogenesis can take 4 months or more. Another advantage of shoot morphogenesis is that the explants are derived from seedling explants, and hence has no need to maintain a constant source of fruiting plants for

explants as in the use of somatic embryo system.

Although shoot morphogenesis and somatic embryogenesis are very different processes, both systems exhibited cultivar-specific responses where some lines are more responsive to tissue culture manipulations than the others.

1.2.3.3 Soybean transformation systems

Several criteria are important for successful transformation of plants including: (i) competence of target tissues for propagation and regeneration; (ii) efficiency of DNA delivery; (iii) method of selection of transgenic tissues; (iv) frequency of recovery of fertile transgenic plants; (v) virulence of the *Agrobacterium* system. Other factors such as simplicity, reproducibility, costs effectiveness and turnover time (to avoid somaclonal variation and possible sterility during culturing) should also be considered.

Three techniques currently used in soybean transformation are *Agrobacterium*-mediated transformation, particle gun bombardment and protoplast transformation.

***Agrobacterium*-mediated transformation**

The major obstacle of *Agrobacterium*-mediated soybean transformation is tissue and host incompatibilities, although this problem can be partially overcome by using highly virulent *Agrobacterium* strains together with the chemical acetosyringone (a phenolic inducer for the DNA transfer process). Therefore, although soybean is a suitable host for *Agrobacterium*, it is clearly not as responsive to infection as many other dicotyledonous plants and the subsequent DNA transfer is less efficient. Futhermore, embryos and embryogenic tissues are typically not very susceptible to *Agrobacterium* infection.

The first successful *Agrobacterium*-mediated soybean transformation was

reported in 1988 (Hinchee, *et al.*, 1988). Cotyledonary node explants from the cultivar Peking were inoculated with *Agrobacterium* containing a T-DNA conferring which conferred kanamycin and glyphosate resistance and expressing β-glucuronidase (GUS). After inoculation, the explants were placed on a medium containing BAP to induce shoot morphogenesis. Kanamycin was also added to select for successful transformants. A few months later, 6% of the selected regenerated plantlets were tested positive for either GUS expression and/or glyphosate tolerance. Genetic analysis of progenies produced from two of these plants displayed Mendelian inheritance suggesting that the introduced DNA was inserted into a single site.

The *Agrobacterium*-mediated transformation on cotyledonary node also successfully introduced the Bean Pod Mottle Virus coat protein into soybean. In this experiment, six independent transformants were recovered from several hundred explants (Di, *et al.*, 1996).

In 1989, transformation of germinating seeds of soybean with *Agrobacterium* was proven to be successfully (Chee, *et al.*, 1989). The method used for transformation required no tissue culture procedures. The plumule, coyledonary node and adjacent cotyledon tissues of germinating seeds were inoculated with *Agrobacterium*. Sixteen putative transformants showed some expression of the transgene. The primary transformation frequency was estimated to about 0.7%. However, only one-tenth of these primary transgenic plants yielded progenies stably expressing the target gene.

Primary transgenic soybean plants were also recovered through *Agrobacterium*-mediated transformation of immature cotyledon tissue. Three transformants were recovered among a total of 18 plants regenerated by somatic embryogenesis after cocultivation with *Agrobacterium* strains carrying a gene encoding the protein 15kDa zein. However, all progenies of the primary transformants did not possess the transgene. Since the origin of primary embryos in soybean could be subepidermal and multicellular, it was believed that the primary

transformants were chimeric (reviewed by Trick and Finer, 1997).

In 1997, Santarem and co-workers successfully employed sonication to enhance *Agrobacterium*-mediated transformation on soybean immature cotyledon explants (Santarem, *et al.*, 1997). The cell-wall disruption caused by the lower energy ultrasound was apparently useful for *Agrobacterium*-mediated transformation. The wounding leads to the production of signal phenolics and enhanced the accessibility of putative cell-wall binding factor to bacterium. The utility of sonication assisted *Agrobacterium*-mediated transformation (SAAT) on embryongenic suspension cultures resulted in the recovery of several stably transformed, non-chimeric clones (Trick and Finer, 1997).

Particle gun bombardment

Particle gun bombardment is the delivery of microprojectiles, usually fine tungsten or gold powder coated with DNA and propelled into the target cells by acceleration. The acceleration can be provided by gunpowder, gases (such as helium or CO_2) or electric discharge. The major advantage of particle gun over *Agrobacterium* is the removal of biological incompatibilities. The success depends critically upon the stability of the target tissue to proliferate and give rise to a fertile plant.

The first report of particle gun-mediated transformation of soybean used shoot meristems as the target tissue. The transformed shoot apices were induced to form multiple shoots. All of the transformed shoots from bombarded meristems were chimeric due to the nature of the target tissue and the method for proliferation and regeneration. However, in subsequent study, non-chimeric transgenic plants were obtained by selecting plants that contained transgenic germ-line cells. However, shoot apex transformation was very labor-intensive, and the meristematic tissue is very difficult to target (Christou, 1990).

Protoplast transformation

In 1988, Wei and Xu described the first report of soybean plant regeneration from protoplast utilized immature cotyledons of soybean cultivar Heilong no. 26 as a protoplast source (Wei and Xu, 1990). Based on this result, Dhir and co-workers produced transgenic soybean via protoplast electroporation. The main benefit of electroporation-mediated gene transfer was the large number of transformants obtained and the production of non-chimeric cell lines. However, soybean protoplast regeneration system was difficult to reproduce (Dhir, *et al.,* 1991*)*.

1.3 Targeted Pharmaceutical and Agricultural Proteins: Lymphocytic Choriomeningitis Virus Nucleoprotein and Goldfish Growth Hormones I and II

In this research, the production platforms of two categories (pharmaceutical and agricultural) of proteins were tested, including the nucleoprotein of lymphocytic choriomeningitis virus (LCMV-NP) and growth hormones I and II of goldfish, *Carassius auratus*.

1.3.1 Production of pharmaceutical proteins

In the past 50 years, notable advances have been made in public health, and reduced the incidence of and the mortality rate associated with infectious diseases. The development of safe and effective vaccines has been playing an important role. For example, vaccination programs worldwide have reduced the incidence of measles and poliomyelitis in developed countries. However, the high production and distribution costs of many vaccines have prohibited their extended applications in developing countries. Thus, new vaccines with low production and distribution costs are imperative (Mason and Arntzen, 1995). The idea of edible vaccine is formulated for achieving this end. In order to test the feasibility of producing antigen in plant

system, nucleoprotein of lymphocytic choriomeningitis virus was chosen to express in *A. thaliana*.

1.3.1.1 Lymphocytic choriomeningitis virus

Lymphocytic choriomeningitis virus (LCMV) is a prototypic member of *Arenaviridae*, a family of enveloped negative-stranded RNA viruses that includes Lassa fever virus and the New World arenaviruses Pichinde, Tacaribe, Machupo and Junin (Auperin, *et al.*, 1989). LCMV gained much scientific attention as an experimental model for the study of acute and persistent infections and the role of immune system in viral pathogenesis. The physical diameter of this virus is in the range of 50-150 nm. Its genome consists of two segments of single-stranded RNA: large and small segments. The small RNA was shown to be uniquely arranged in a pattern termed 'ambisense' (i. e. coding regions were found in both sense and antisense orientations of the mRNA). It encodes three major structural proteins: the internal nucleoprotein (NP; MW 63, 000) which is associated with the genomic RNA, and the two glycoproteins GP-1 (MW 43, 000) and GP-2 (MW 36, 000) which are derived from a common precursor, GP-C. The NP-encoding sequence of the 3' half of the molecule is viral complementary and the GP-C-encoding 5' half is of viral sense. The large segment encodes a high-molecular weight protein (approximately 200, 000) which is thought to be part or all of the viral RNA-dependent RNA polymerase (Auperin, *et al.*, 1989; Fuller-pace and Southern, 1988).

Arenaviruses are zoonatic (i. e. found in animals). Each virus is associated with either one species or a few closely related rodents, which constitute the 'natural reservoir of virus'. The LCMV is associated with the Old World rats and mice (family *Muridae*, subfamily *Murinae*). These rodents colonize in Europe, Asia, Africa and America (Jahrling and Peters, 1992).

The rodent hosts are chronically infected with arenaviruses without causing a severe illness. LCMV appears to be passed from mother rodents to their offsprings during pregnancy, and thus remains in the rodent population to generation and

generation. The virus is shed into the environment via the urine or droppings of the infected hosts (Barton and Mets, 1999).

Human infection by arenaviruses such as LCMV is incidental to the natural cycle of viruses and occurs when an individual comes in contact with the excretions or materials contaminated with the excretions of infected rodents. Infection can also occur by inhalation of tiny particles soiled with rodent urine or saliva (aerosol transmission) (Barton and Mets, 1999).

The pathogenicity of LCMV is very diverse. Related clinical manifestations included mild influenza-like illness or occasionally, meningeal or meningoencephalomyelitic symptoms, orchitis or parotitis, Severe or lethal meningoencephalitis also occurred occasionally. There were also some reports of fetal abortion or malformation caused by transplacental infection (Childs, *et al.*, 1991).

Prenatal LCMV infection was first reported in England in 1955. An infant born 12 days after maternal illness became symptomatic on the 7^{th} day after birth and died 5 days later. LCMV was recovered from the infant's cerebrospinal fluid (Barton and Mets, 1999). Twenty such cases from Germany, Lithuania and France have been reported since 1974 in which seventeen of these had hydrocephalus, and one had microcephalus. Of these eighteen, chorioretinitis was found in sixteen out of the eighteen cases. Although reliable documentation of sequenlae is not available for all twenty cases, spastic tetraparesis persisted in at least eleven. Four of the twenty children died during the first year of life, and three died during the second year (Sheinbergas, *et al.*, 1981). Since 1993, there have been five reports from the U. S. A. documenting the teratogenicity of LCMV in nine infants. Five additional U. S. A. infants were recently diagnosed with this infection (Barton and Mets, 1999).

The true incidence of prenatal LCMV infection, however, may have been underestimated because of its reliance on the complement fixation test, which can be negative even when infection has proven the otherwise. Serological diagnosis of

LCMV infection has recently been improved by the use of the indirect fluorescent antibody (IFA) test and the rapid fluorescent foci inhibition test (RFFIT), and subsequently by the application of enzyme-linked immunosorbent assay (ELISA), and reverse transcription-polymerase chain reaction assay (RT-PCR) of glycoprotein and nucleoprotein genes (Park, *et al.*, 1997; Sheinbergas, *et al.*, 1981).

Lymphocytic choriomeningitis virus has the greatest potential geographic range of any arenavirus, and may occur on all lands where the genus *Mus* (the genus of common house mice) has been introduced. Using ELSIA techniques, prevalence of LCMV infection among human and mice populations were estimated in several cities over the world (summarized in Table 6).

Recently, the unusual transmissible agent of MaTu-MX (associated with MaTu human carcinoma cells) was found to be a new strain of LCMV. Using MX LCMV NP in immunoprecipitation and solid phase radioimmunoassay, prevalence of anti-LCMV antibodies in human sera was estimated to be 37% in Bratislava at Slovakia (Reiserová, *et al.*, 1999).

Table 6 Prevalence tests of serum antibodies against LCMV in several cities over the world.

Cities	**Prevalence (%)**	**Studied samples**	**References**
Birmingham	4.9	human	Stephensen, *et al.*, 1992
San Antonio	2.0	human	Stephensen, *et al.*, 1992
Baltimore	9.0 4.7	mice human	Childs, *et al.*, 1992
Nova Scotia	4.0	human	Marrie and Saron, 1998

It is well-known that LCMV is able to stimulate the mucosal immune system via gastric inoculation. In 1996, Rai and co-workers reported that LCMV could use M-cell transcytosis to traverse the gut. Once LCMV crosses the intestinal epithelia, it can come in contact with macrophages and dendritic cells. This laid the groundwork

for future LCMV oral vaccine development (Rai, *et al.*, 1996).

1.3.1.2 Nucleoprotein of LCMV

In this project, the cDNA clone (1677bp) encoding the full-length NP of LCMV was used. The molecular size of the mature protein is around 63 kDa. The nucleoprotein is an ideal antigen which contains a cytotoxic T lymphocyte-specific (CTL-specific) epitope at residues 117-130 (Martins, *et al.*, 1995).

Nucleoprotein seems to have a more consistent immunogenicity when compared to other antigens such as the glycoprotein. For common experimental mice, both H-2^d and H-2^b haplotypes could recognize nucleoprotein, but only H-2^b haplotypes showed CTL-specific towards glycoprotein (Yokoyama, *et al.*, 1995).

Besides, nucleoprotein was found to be very conserved among LCMV strains and other arenaviruses as shown by aligning the amino acid sequences of the nucleoproteins of the Josiah and Nigerian strains of Lassa virus, the WE and ARM strains of LCMV and the Tacaribe and Pichinde viruses (Table 7) (Auperin and McCormick, 1989; Reiserová, *et al.*, 1999).

Table 7 Amino acid sequence identities between LASSA JOS, LASSA NIG, LCMV WE, LCMV ARM and Tacaribe and Pichinde.

	LASSA JOS	**LASSA NIG**	**Tacaribe**	**Pichinde**
LCMV ARM	**58%**	**57%**	**46%**	**46%**

The epitope region (at amino acid residues 117 to 130) of the nucleoproteins among them are also similar, as illustrated in Table 8 (Reiserová, *et al.*, 1999).

Table 8 Epitopes of nucleoproteins of arenaviruses.

LCMV WE	**ERPQTSGVYMGNLT**
LCMV ARM	**ERPQTSGVYMGNLT**
LASSA JOS	**ERP**LSA**GVYMGNL**S
LASSA NIG	**ERP**LS**SGVYMGNL**S
Tacaribe	**E**-SNGTNA**YMGNL**P
Pichinde	**E**GLSQP**GVYE**G**NL**T

The amino acid sequence identity may allow cross immunoresponse triggered by LCMV-NP to other related strains (Auperin and McCormick, 1989; Reiserová, *et al.*, 1999). It thus has a high potential to be developed into a broad range vaccines (Castrucci, *et al.*, 1994; Hassett, *et al.*, 1997; Martins, *et al.*, 1995; von Herrath, *et al.*, 1999; von Herrath, *et al.*, 2000; Yokoyama, *et al.*, 1995).

1.3.2 Production of agricultural proteins

For production of agricultural proteins, growth hormones (GHI and GHII) of goldfish, *Carassius auratus*, were chosen as the targets. Feed can account for up to 50% of the operating cost of commercial fish production. Any improvement in growth rate of fish will significantly reduce expenses in terms of time, labor, feed and space (Cavari, *et al.*, 1993). Thus, growth hormones produced in transgenic plants may be used to promote the growth of fish via feeding.

1.3.2.1 *Carassius auratus*

Goldfish, *Carassius auratus*, belongs to the *Cyprinidae* family. It has a great aquacultural importance in South China regions. Besides, it is also a good experimental fish model because of its small size, low cost and ease of cultivation. It is generally fed with dried flake or pellet food. In aquarium, goldfishes are not particularly aggressive and the combing size is not often a problem.

1.3.2.2 Growth hormones I and II

Growth hormones are originated from adenohypophysis of pituitary and their target sites include most of the body tissue. They stimulate RNA synthesis, protein synthesis and tissue growth, elevate the transport of glucose and amino acids into cells, and increase lipolysis and antibody formation. In fish, growth hormones also influence osmoregulation and reproduction (Corin, *et al.*, 1990; Fine, *et al.*,1993; Mahmoud, *et al.*, 1996). Their releases are stimulated by growth hormone releasing hormone (GRH) and inhibited by somatostatin. For goldfish, gonadotropin-releasing hormone, dopamine, neuropeptide Y, thyrotropin-releasing hormone and cholecystokinin also play important roles in stimulating secretion of growth hormones. Together with anabolic steroid, thyroid hormones, insulin, insulin like growth factor, a group of biological factors influencing normal growth is established (Farmer, *et al.*, 1976; Mahmoud, *et al.*, 1996).

In mammals, growth hormone variants come from differential processing of a single primary RNA transcript. However, the growth hormones I and II found in goldfish (or broadly speaking in teleosts) appear to be products of different genes, as the two growth hormones cDNAs have distinct nucleotide sequences at their coding and 3' untranslated regions. Both growth hormones consist of 210 amino acids including a putative signal peptide of 22 amino acids. For cDNA encoding growth hormone I, it gives a polypeptide with five cysteine residues (possessing an additional cysteine at position 123 that is not found in the growth hormone of most teleost). For growth hormone II that contains four cysteine residues, two intramolecular disulfide bonds will be formed. The additional cysteine in growth hormone I may be involved in receptor binding in common carp (Law, *et al.*, 1996). The nucleotide sequences of growth hormones I and II are very similar (around 93% homology) while the homology of predicted amino acid sequences is up to 94%. Growth hormone I and II cDNA used to construct the transgenic plants were 1117bp and 1179bp in sizes, respectively. Both of the predicted polypeptides will have a molecular size of approximately 23kDa (Law, *et al.*, 1996).

Nevertheless, the actual differences in the biochemical and biological properties between the two growth hormones are not very clear. There was report showing that the two growth hormone variants in Atlantic cod, differing by one amino acid, exhibited different biochemical and immunological characteristics in SDS-polyacrylamide gel electrophoresis and radioimmunoassay (Mahmoud, *et al.*, 1996; Law, *et al.*, 1996).

As mentioned above, the ultimate goal of expressing growth hormones I and II in transgenic plants is to promote fish growth by feeding. This idea is supported by several reports showing positive effects of exogenous growth hormone on fishes (Table 9).

The effects on bovine growth hormone and muscle composition of coho salmon by intraperitioneal injection of a pellet implantation of exogenous growth hormone were studied (Higgs, *et al.*, 1975). Mean weights of fishes treated for 8 weeks were 40-66% higher than the control. In addition, biologically active polypeptide hormones were also produced in bacteria by expressing the corresponding gene. When the bacterial extract was given to yearling fishes via intraperitoneal injections, significant weight increases were observed in treated group when compared with control group (Agellon, *et al.*, 1987). Furthermore, dip method was also developed to promote the growth of fish (Higgs, *et al.*, 1975). When fries of rainbow trout were given a single treatment of the recombinant growth hormone, a significant difference in weight was again observed between the control and growth hormone treated group (Table 9).

Table 9 Effect of exogenous growth hormones on fish growth.

Year	Major Findings	References
1975	Weight gain in coho salmon after implanting bovine GH/ cholesterol pellet	Higgs, *et al.*, 1975
1980	Mammalian growth hormone is effective in increasing growth rate of killifish	Prack, *et al.*, 1980
1981	Increases in body size and weight of juvenile rainbow trout resulted from injection of purified chum salmon growth hormone	Cavari, *et al.*, 1993
1987	Promotion of rapid growth of rainbow trout (*salmo gairdneri*) by feeding with a recombinant fish growth hormone produced in *E. coli*	Agellon, *et al.*, 1987

1.4 Hypothesis and Objectives

Based on the findings and reports mentioned above, I hypothesize that transgenic plant systems can effectively produce high-valued foreign proteins, especially in plants with protein-rich seeds. This hypothesis was tested using two integral approaches: 1) transfer the target recombinant DNA constructs into the model plant system, *Arabidopsis thaliana*, for quick assessment of the expression levels and production efficiency; and 2) optimize a transform systems in the seed protein-rich plant soybean.

The specific objectives of this thesis are: 1) to study the expression of agricultural important (goldfish growth hormones I and II) and pharmaceutical proteins (Lymphocytic choriomeningitis virus nucleoprotein) in the model plant *Arabidopsis thaliana*; 2) to optimize the protocol for soybean transformation.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Plants, bacterial strains and vectors

The *Escherichia coli* strains DH5α and the plasmid pBluescript KSII(+) were used as the host and vector, respectively, for gene cloning unless stated the otherwise. For vacuum infiltration transformation of *A. thaliana* ecotype Columbia-0 (Col-0), the *Agrobacterium tumefaciens* strain GV3101 containing helper Ti-plasmid pMP90 was employed as the bacterial host for the target genes. LCMV-NP and GHI & II were cloned into the plant expression vectors V7 and W104. For transformation of *G. max* (soybean), *Agrobacterium tumefaciens* strains GV3101 containing the helper Ti-plasmid pMP90 and LBA4404 containing the Ti-plasmid pAL4404 were adopted to transform soybean varieties Ji lin xiao li no. 1, He feng no. 25, Ji lin no. 30, Ji lin no. 36, Chang nong no. 5, Zhe chun no.3 and Ai jiao zao. The reporter gene (GUS) was placed within the T-DNA of the expression vector pBI121. Bacterial production of the recombinant LCMV-NP was produced by a His-tag system using the plasmid pET-30a-(+) and the bacterial host DE3. The plasmid system pGEM-3Zf(+) was used in the *in vitro* transcription process. A list of plant hosts, bacterial strains, plasmid vectors used in the research was shown in Table 10.

Table 10 Plants, bacterial strains and vectors used.

Bacteria/ Plasmid	**Description**	**References**
Agrobacterium tumefaciens GV3101/ pMP90	For *A. thaliana* and *G. max* transformation	Koncz and Sdell, 1986.
Agrobacterium tumefaciens, LBA4404/ pAL4404	For *G. max* transformation	Hoekema, *et al.*, 1983
DE3	For LCMV-NP protein production in bacterial system	Phillips, *et al.*, 1984; Wood, *et al.*, 1966.
Escherichia coli DH5α	For regular gene cloning	Lab stock
Plant hosts	**Description**	**References**
Columbia-0	*A. thaliana* ecotype for transformation	Lab stock
HML 554-1	Goldfish GHII heterozygous transgenic *A. thaliana*	From Prof. Hon-Ming Lam and Mr. Jack Lo
HML 554-3	Goldfish GHII heterozygous transgenic *A. thaliana*	From Prof. Hon-Ming Lam and Mr. Jack Lo
HML 554-4	Goldfish GHII heterozygous transgenic *A. thaliana*	From Prof. Hon-Ming Lam and Mr. Jack Lo
HML 555-1	Goldfish GHI heterozygous transgenic *A. thaliana*	From Prof. Hon-Ming Lam and Mr. Jack Lo
HML 555-3	Goldfish GHI heterozygous transgenic *A. thaliana*	From Prof. Hon-Ming Lam and Mr. Jack Lo
HML 555-7	Goldfish GHI heterozygous transgenic *A. thaliana*	From Prof. Hon-Ming Lam and Mr. Jack Lo
HML 554 1-3	Goldfish GHII homozygous transgenic *A. thaliana*	This work
HML 554 3-1	Goldfish GHII homozygous transgenic	This work

	A. thaliana	
HML 554 3-3	Goldfish GHII homozygous transgenic *A. thaliana*	This work
HML 554 3-4	Goldfish GHII homozygous transgenic *A. thaliana*	This work
HML 554 4-1	Goldfish GHII homozygous transgenic *A. thaliana*	This work
HML 554 4-5	Goldfish GHII homozygous transgenic *A. thaliana*	This work
HML 555 1-3	Goldfish GHI homozygous transgenic *A. thaliana*	This work
HML 555 1-6	Goldfish GHI homozygous transgenic *A. thaliana*	This work
HML 555 3-4	Goldfish GHI homozygous transgenic *A. thaliana*	This work
HML 555 7-4	Goldfish GHI homozygous transgenic *A. thaliana*	This work
HML 603 A-1	LCMV-NP heterozygous transgenic *A.thaliana*	This work
HML 603 A-3	LCMV-NP heterozygous transgenic *A.thaliana*	This work
HML 603 C-2	LCMV-NP heterozygous transgenic *A.thaliana*	This work
HML 603 D-1-1	LCMV-NP heterozygous transgenic *A.thaliana*	This work
HML 603 D-1-2	LCMV-NP heterozygous transgenic *A.thaliana*	This work
HML 603 D-1-3	LCMV-NP heterozygous transgenic *A.thaliana*	This work
HML 603 D-1-4	LCMV-NP heterozygous	This work

	transgenic *A.thaliana*	
HML 604 C-1-1	LCMV-NP heterozygous transgenic *A.thaliana*	This work
HML 604 C-1-2	LCMV-NP heterozygous transgenic *A.thaliana*	This work
HML603C-2-G	LCMV-NP homozygous transgenic *A.thaliana*	This work
HML603D-1-2-E	LCMV-NP homozygous transgenic *A.thaliana*	This work
HML603D-1-2-G	LCMV-NP homozygous transgenic *A.thaliana*	This work
HML603D-1-2-I	LCMV-NP homozygous transgenic *A.thaliana*	This work
HML603D-1-4-M	LCMV-NP homozygous transgenic *A.thaliana*	This work
HML603D-1-4-N	LCMV-NP homozygous transgenic *A.thaliana*	This work
HML604C-1-1-A	LCMV-NP homozygous transgenic *A.thaliana*	This work
HML604C-1-1-B	LCMV-NP homozygous transgenic *A.thaliana*	This work
HML604C-1-1-G	LCMV-NP homozygous transgenic *A.thaliana*	This work
HML604C-1-1-K	LCMV-NP homozygous transgenic *A.thaliana*	This work
HML604C-1-1-O	LCMV-NP homozygous transgenic *A.thaliana*	This work
Ai jiao zao	Soybean variety for transformation	Collection of CAAS
Chang nong no. 5	Soybean variety for transformation	Collection of CAAS
He feng no. 25	Soybean variety for transformation	Collection of CAAS
Ji lin no. 30	Soybean variety for transformation	Collection of CAAS
Ji lin no. 36	Soybean variety for transformation	Collection of CAAS
Ji lin xiao li no. 1	Soybean variety for	Collection of CAAS

	transformation	
Zhe chun no.3	Soybean variety for transformation	Collection of CAAS
Plasmid vectors	**Description**	**References**
pBI121	Plasmid carrying GUS reporter gene	Clontech Inc., Palo Alto, CA, U.S.A.
pBluescript II KS (+)	Plasmid for subcloning of target gene	Strategene, La Jolla, CA, U.S.A.
pBluescript II KS (-)-GHI	Plasmid containing GHI	Law, *et al.*, 1996.
pBluescript II KS (-)-GHII	Plasmid containing GHII	Law, *et al.*, 1996.
pET-30a-c(+)	Plasmid for LCMV-NP protein expression in bacterial system	Rosenberg, *et al.*, 1987; Studier, *et al.*, 1990.
pGEM-3Zf (+)	Plasmid for *in vitro* transcription	Promega Biosciences, Hercules, CA, U.S.A.
pUC-NP	Modified pUC plasmid containing LCMV-NP encoding gene	Stock from Dr. M. F. Saron at Pasteur Institute
V7 plant expression vector	Plasmid for plant transformation of target genes	Brears, *et al.*, 1993.
W104 plant expression vector	Plasmid for plant transformation of target genes	Brears, *et al.*, 1993.
pET-30a (+)-LCMV-NP	Protein expression vector in bacterial system containing LCMV-NP cDNA	This work
pGEM-3Zf(+)-GHI	Plasmid containg GHI cDNA for *in vitro* transcription	This work
pGEM-3Zf(+)-GHII	Plasmid containg GHII cDNA for *in vitro* transcription	This work
pGEM-3Zf(+)-LCMV-NP	Plasmid containg LCMV-NP cDNA for *in vitro* transcription	This work
pBluescript II KS	Plasmid containing	This work

(+)-LCMV-NP	LCMV-NP cDNA for generation of cRNA probes	
V7-GHI	Plant expression vector containing GHI cDNA	This work
V7-GHII	Plant expression vector containing GHI cDNA	This work
W104- LCMV-NP	Plant expression vector containing LCMV-NP cDNA	This work

2.1.2 Chemicals and Regents

Regular chemicals were purchased from Sigma-Aldrich Co. (Saint Louis, MO, U.S.A.). Organic solvents were from Merck & Co., Inc. (New Jersey, U.S.A.). Bacterial growth media were from Difco (Sparks, MD, U.S.A.) and Murashige & Shoog (MS) salt mixture was from Gibco BRL (Grand Island, NY, U.S.A.). Phenolic compound, phytohormones and antibiotics used in bacteria and plant cultures were purchased from Aldrich Chem. (Saint Louis, MO, U.S.A.), Amresco Inc. (Solon, OH, U.S.A.) and Sigma-Aldrich Co. (Saint Louis, MO, U.S.A.). Silwet-77 for plant transformation experiment was from Lehle seeds (Round Rock, TX, U.S.A.). Metro-mix-200 soil for plant growth was from Hummert International Horticultural Supplier (Earth City, MO, U.S.A.). Chemicals for gel electrophoresis were from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Restriction enzymes were from New England Biolabs. Inc. (Beverly, MA, U.S.A.) and Promega Biosciences (San Luis Obispo, CA, U.S.A.). Other enzymes for molecular biology experiments and reagents for DNA and RNA manipulation and detection were from Roche Diagnostic limited (Basel, Switzerland) (Appendix I). Positively charged nylon membrane for Northern and Southern blot experiments and Sequi-Blot^{TM} PVDF membrane for

Western blot experiment were from Roche Diagnostic limited (Basel, Switzerland) and Bio-Rad Laboratories (Hercules, CA, U.S.A.), respectively. Talon metal affinity resins were from Clontech Laboratories, Inc. (Palo Alto, CA, U.S.A.). Bio-MAX X-ray film was from Eastman Kodak (Rochester, NY, U.S.A.). Detailed information on chemical used was listed in Appendix II.

2.1.3 Commercial kits

The following reagent kits were used in this research (Table 11) (For details, please see Appendix III).

Table 11 Commercial kits used.

Kits	Experiments	Company
ABI prism dRhodamine terminator cycle sequencing ready reaction kit	DNA sequencing	Applied Biosystems (Foster City, CA, U.S.A.)
Aurora™ western blot chemiluminescent detection system	Western blot analysis	ICN Biomedicals, Inc. (Costa Mesa, CA, U.S.A.)
Bio-rad Prep-A-Gene DNA Purification kit	Target genes subcloning	Bio-Rad Laboratories (Hercules, CA, U.S.A.)
Bio-Rad protein assay kit	Estimation of concentration of plant protein extract	Bio-Rad Laboratories (Hercules, CA, U.S.A.)
Clone checker system	Target genes subcloning	GibcoBRL (Grand Island, NY, U.S.A.)
DIG detection system (CSPD, ready-to-use and Anti digoxigenin-AP, Fab fragments)	Southern and Northern blot analyses	Roche Diagnostic limited (Basel, Switzerland)
DIG RNA labeling kit	Generation of DIG-labelled cRNA	Roche Diagnostic limited (Basel, Switzerland)

	probes	
High pure PCR product purification kit	Target genes subcloning	Roche Diagnostic limited (Basel, Switzerland)
MabTrap G II	Purification of anti-LCMV polyclonal antibodies	Amersham Pharmacia. Biotech. (Buckinghamshire, England)
Rabbit reticulocyte lysate system	*In vitro* translation	Promega Biosciences (San Luis Obispo, CA, U.S.A.)
Ribomix large scale RNA production system-T7	*In vitro* transcription	Promega Biosciences (San Luis Obispo, CA, U.S.A.)
Ribomix large scale RNA production system-SP6	*In vitro* transcription	Promega Biosciences (San Luis Obispo, CA, U.S.A.)
Transcend non-radioactive translation detection system (Chemiluminescent)	Detection of *in vitro* transcripted and translated products	Promega Biosciences (San Luis Obispo, CA, U.S.A.)
Wheat germ extract system	*In vitro* translation	Promega Biosciences (San Luis Obispo, CA, U.S.A.)
Wizard plus minipreps DNA purification kit	Target genes subcloning	Promega Biosciences (San Luis Obispo, CA, U.S.A.)

2.1.4 Primers and Adaptors

All primers were bought from Integrated DNA Technologies, Inc (Coralville IA, U.S.A.). A full list of their sequences was shown Table 12.

Table 12 Primers and adaptors used.

Primer name	**Sequence (5' to 3')**	**Use for**
35S promoter sequencing primer	TCCAACCACGTCTTCAAAGC	Sequencing
T3 primer	AATTAACCCTCACTAAAGGG	Sequencing
T7 primer	GTAATACGACTCACTATAGGGC	Sequencing
HMOL 532	AATTGCGGCCGC	Generation of adapter with *Xho*I and *EcoR*I sites
HMOL 533	TCGAGCGGCCGC	Generation of adapter with *Xho*I and *EcoR*I sites
HMOL 534	GCGGCCGCAAGCTT	Generation of adapter with *Xba*I and *Bbu*I sites
HMOL 535	CTAGAAGCTTGCGGCCGCCATG	Generation of adapter with *Xba*I and *Bbu*I sites
HMOL 538	AAACATATGCCATGTCCTTGTC TAAGGAAGTTAAG	PCR screening of LCMV-NP clones
HMOL 539	AGGATCTGAGATCTTTGGTCTAG	PCR screening of LCMV-NP clones
HMOL 540	TGCGCAACCCGGGTTGACCTC	PCR screening of LCMV-NP clones
HMOL 541	AAAGTATACTTAGAGGTGTCACAA CATTTGGGCC	PCR screening of LCMV-NP clones
HMOL 643	GATATCGGATCCATGTCCTTGTCT AAGG	Amplification of LCMV-NP with *BamH*I at 5' end
HMOL 646	GAGCTCGAGCTCTTAGAGTCTCAC AAC	Amplification of LCMV-NP with

		*Sac*I at 3'end
HMOL 703	CACACCGATACCATCAGCGATC	PCR screening for GUS transgene
HMOL 704	TCACCGAAGTTCATGCCAGTCC	PCR screening for GUS transgene
HMOL 705	AGATTGGGGTTTGTTTCGAGG	PCR screening for *virA* gene
HMOL 706	CCACGCGGTGTTTTACAGG	PCR screening for *virA* gene

The two adaptors used to subclone LCMV-NP cDNA insert into V7 and W104 plant expression vector were synthesized by pairing HMOL 532 and HMOL 534 with HMOL 533 and HMOL 535, respectively, by first incubated them at 94°C 5 minutes, and then gradually decreased the incubation temperature to 0°C.

2.1.5 Equipments and facilities used

All equipments and facilities were provided by Department of Biology, CUHK. An inventory is shown Appendix IV.

2.1.6 Buffer, solution, gel and medium

Unless otherwise stated, buffer, solution and medium were prepared according to the formulation listed in Appendix V.

2.2 Methods

2.2.1 Molecular Techniques

2.2.1.1 Bacterial cultures for recombinant DNA and plant transformation

Bacterial strains (*E. coli* and *A. tumefaciens*) were inoculated from glycerol stocks (stored at -70°C) into LB broth (*E. coli*) or YEP broth (*A. tumefaciens*) and shake at 200 rpm (Orbital shaker, Lab. line 4628-1), 37°C, for overnight (*E. coli*) or at 250 rpm, 28°C, for two days (*A. tumefaciens*).

Antibiotics were added when appropriate to the growth media in final concentrations of 100mg/ L, 50mg/ L, 50mg/ L, 25mg/ L, 50mg/ L for ampicillin, kanamycin, rifampcin, gentamycin and streptomycin, respectively.

2.2.1.2 Recombinant DNA techniques

Restriction of DNA was normally done with 2 units of restriction enzyme per microgram of DNA in the presence of 1g/L Bovine serum albumin (BSA) in 1X restriction buffer (as recommended in the company product notes) and incubated at 37°C for 3 hours to 16 hours. Ligation of DNA fragments and vectors with compatible ends were performed with 1mM dATP and 3 units T4 DNA ligase (Promega) in 1X T4 DNA ligase buffer (as recommended in the company product notes) and the reaction mixes were incubated at 16°C overnight. For blunt end ligation, the ligation was performed with 0.5mM dATPs, 150mM sodium chloride and 50 Weiss units/ml T4 DNA Ligase (Promega) at 22.5°C overnight.

DNA purification was carried out as described in the company manuals using commercial kits, such as Bio-Rad Prep-A-Gene DNA Purification kit and High Pure PCR Product Purification Kit.

2.2.1.3 Preparation and transformation of DH5α, DE3 and *Agrobacterium* competent cells

(i) Preparation of $CaCl_2$-competent DH5α and DE3 competent cells

DH5α or DE3 cells was first inoculated into 5 ml LB medium and shook at 200 rpm (Orbital shaker, Lab. line 4628-1), 37°C overnight to make a starter culture. Four millilitres starter culture was then added to 400 ml LB medium and allowed to grow to until optimal density at 600nm (O.D. 600) reached 0.4. The culture was chilled on ice for 10 minutes and centrifuged at 1600g for 7 minutes at 4°C. After discarding the supernatant, the cell pellet was resuspended in 80ml ice-cold 60mM $CaCl_2$ solution. The cell culture was centrifuged at 1100g for 5 minutes at 4°C. Supernatant was discarded again and cell pellet was resuspended with 80ml ice-cold 60mM $CaCl_2$ solution. The suspension was allowed to stand on ice for 30 minutes. It was then centrifuged at 1100g for 5 minutes at 4°C. The pellet was finally resuspended with 8ml ice-cold $CaCl_2$ solution after discarding the supernatant. Aliquots of 0.1 ml were transferred into prechilled, sterile 1.5ml eppendorf tubes and stored at -70°C until ready to use (modified from Sambrook, *et al.*, 1989).

(ii) Transformation of DH5α and DE3 competent cells

The plasmid DNA was transformed into DH5α or DE3 competent cells via heat shock calcium chloride mediated transformation. The plasmid DNA was added to an aliquot of 0.1 ml pre-chilled competent cells. The mixture was allowed to sit on ice for 10 minutes, followed by a heat shock at 42°C was carried out for 2 minutes. Cells were immediately rescued by adding 0.5 ml LB broth and incubated at 37°C for 1 hour with shaking at 150 rpm (Orbital shaker, Lab. line 4628-1). The recovered cells were spread on LB agar plate with appropriate antibiotic for selection and incubated at 37°C overnight.

(iii) Preparation of *Agrobacteria* Electro-competent *A. tumefaciens* cells

GV3101/ pMP90 or LBA4404/ pAL4404 was inoculated into 10ml YEP broth supplemented with antibiotics when appropriate and shook at 250 rpm (Orbital shaker, Lab. line 4628-1), 28°C, overnight to prepare a starter culture. Eight millilitres of the dense culture was then inoculated into 400ml YEP medium also supplemented with appropriate antibiotics was allowed to grow until O.D.600 was between 0.5 to 1.0. The culture was harvested by chilling on ice for 15 minutes and centrifuged at 4000g for 15 minutes at 4°C. After discarding the supernatant, the cell pellet was resuspended with 400ml ice cold sterile deionized water. The resuspension was centrifuged at 4000g for 15 minutes at 4°C again. The pellet was then resuspended with 200ml ice cold sterile deionized water after the removal of supernatant. The concentrated suspension was spun at 4000g for 15 minutes at 4°C. After discarding the supernatant, the cell pellet was resuspended with 4ml 10% filter sterile (with 0.2μm filter) glycerol. The 4ml glycerol suspension was again

centrifuged at 4000g for 15 minutes at 4°C. Finally, the cell pellet was resuspended in 0.4ml ice cold 10% sterile glycerol after the removal of the supernatant. Forty microlitres aliquots were aspirated into prechilled, sterile 1.5ml eppendorf tubes and stored at -70°C until ready to use (Dower, *et al.*, 1992).

(iv) Transformation of electro-competent *Agrobacterium* cell

An aliquot of 40µl of electro-competent *Agrobacterium* strain, GV3101/pMP90 or LBA4404/ pAL4404, was thawed on ice and mixed with 10ng of recombinant plasmid in a pre-chilled electroporation cuvette. The mixture was further incubated on ice for 30 minutes. After drying the cuvette with tissue paper to remove moisture on the surface, the cuvette containing sample mixture was inserted into the gene pulser apparatus (BioRad GenePulser, Model No. 165-2076). Electroporation was performed at: 25 µF, 2.5 kV and 600 ohms. After discharge, 1ml SOC medium was immediately added to rescue the cells. The culture was then transferred to 1.5 ml eppendorf and incubated at 28°C for 2 hours with shaking at 200 rpm (Orbital shaker, Lab. line 4628-1). The recovered culture was then spreaded on YEP agar plate supplemented with appropriate antibiotics (GV3101/pMP90: 50 mg/ L rifampicin, 25 mg/ L gentamycin; LBA4404/ pAL4404: 50mg/ L streptomycin; *Agrobacterium* containing V7 and W104 or pBI121 vectors: 50 mg/ L kanamycin) and allowed to grow at 28°C for 2-3 days.

2.2.1.4 Gel electrophoresis

(i) DNA gel electrophoresis

Agarose gel was prepared by heat-dissolving 8mg/ ml agarose powder in 1X TAE using a microwave. After cooling down to below 70°C, 1μl 1mg/ ml ethidium bromide was added before pouring onto a gel caster. DNA samples in 1X bromophenol blue loading dye were normally loaded onto 0.8% agarose gels. The gel electrophoresis was run in 1X TAE buffer at 80 V for 30 minutes to 3 hours.

(ii) RNA denaturing gel electrophoresis

One percent denaturing gel was prepared by heat-dissolving 1g agarose gel powder in 87 ml DEPC-treated deionized water using a microwave. Ten millilitres 10X MOPS buffer and 3 ml formaldehyde (37%, pH≥4.0) were added after the temperature was cooled to below 70°C. The denaturing gel solution was then mixed and poured in a clean gel tray. Twenty microgram aliquots of RNA sample was added in a final volume of 35μl solution containing 3.5 μl 10X MOPS, 17.5 μl 37% formamide, 6.13 μl formaldehyde, 1μl 1mg/ ml ethidium bromide and 1 μl 6X bromophenol blue loading dye. The sample mixture was denatured at 55°C for 20 minutes and then put immediately onto ice. The denatured RNA samples were loaded onto the denaturing agarose gel and gel electrophoresis was run in 1X MOPS buffer at 80 V for 2 hours.

(iii) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gel electrophoresis adopted was a discontinuous system, including stacking gel (pH6.8) and separation gel (pH8.8). Acrylamide mixture of separation gel was first poured into the plate and spacer assemblage after the addition of TEMED and ammonium persulfate as mentioned Section 2.1.6. Stacking gel was added after the separation gel was set (formula for preparation of separation and stacking gels were described in Appendix).

Protein samples in 1X SDS sample buffer were loaded onto the gel after heating at 90-100°C for 2 minutes. Electrophoresis was carried out at 20mA in glycine running buffer and was usually performed until the bromophenol blue dye had run off the bottom of the gel.

2.2.1.5 DNA, RNA and protein extractions

(i) DNA extraction from plant tissue

The protocol of isolation of plant genomic DNA was modified from Doyle and Doyle (1987). Approximately 1g plant tissue was first frozen and ground in liquid nitrogen before homogenized with 5ml 2X CTAB extraction buffer. The extract was then incubated at 60°C for 30 minutes before centrifuged at 3000g at room temperature for 10 minutes. Aqueous layer was transferred to a new tube and extracted with phenol: chloroform: isoamylalcohol (PCI) (25:24:1) once and chloroform: isoamylalcohol (CI) (24:1) for twice. Ethanol precipitation of nucleic acid was done by adding one-tenth volume of 3M sodium acetate (pH 5.2) and 2

volumes of absolute ethanol and kept at -20°C overnight. After centrifugation at 10 000g for 15 minutes and discarding the supernatant, the pellet was washed with CTAB washing buffer and air-dried. Finally, the pellet was resuspended in sterilized deionized water supplemented with 1μg/ ml RNaseA and incubated at 37°C for 1 hour to remove RNA (Doyle and Doyle, 1987).

(ii) Plasmid DNA extraction from bacterial cells

Plasmid DNA was isolated using the Wizard plus minipreps DNA purification kit (Promega). The procedures were according to the commercial manuals except the volume of cell culture used. For high copy number plasmids, such as pBluescript, 3ml cell culture was used as starting material per reaction. However, for low copy number plasmids, such as V7 and W104, 20ml cell culture was used instead.

(iii)RNA extraction from plant tissues

Plant RNA extraction protocol was modified from a standard protocol (Ausubel, *et al.*, 1995). Approximately 5g plant tissue for RNA extraction was harvested and immediately frozen and ground in liquid nitrogen before homogenized in 25ml extraction buffer. The aqueous portion of the sample was then extracted twice with PCI followed by two rounds of CI extraction. One-tenth volume of 3M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol were added to the resulting aqueous layer and the sample was stored at -20°C overnight to precipitate the nucleic acids. After centrifugation at 8000rpm for 20 minutes (roter F34-6-38: Centrifuge 5810R, Eppendorf), supernatant was discarded and the nucleic acid pellet

was resuspended with 1 ml 3M sodium acetate, pH 5.6 and the suspension was transferred to a 1.5ml microcentrifuge tube. After centrifugation at 13000 rpm for 30 minutes, mRNA and rRNA were precipitated and tRNA and DNA remained in the supernatant. After repeating the 3M sodium acetate pH5.6 extraction one more time (using 0.5ml this time), the pellet was then resuspended in 0.4ml 0.3M sodium acetate pH5.6 and the RNA was precipitated by adding 1ml 100% ethanol and kept at -20°C overnight. After centrifugation at 13, 000 rpm (Refrigerated centrifuge 5810R, Eppendorf 03463) for 30 minutes and removal of supernatant, the RNA pellet was air-dried before resuspended in DEPC-treated deionized water.

(iv) Protein extraction from plant tissues

Two grams of transgenic plant tissue was ground in 5 ml ice-cold 1X PBS extraction buffer (the composition of 1X PBS extraction buffer for LCMV-NP and GHI & II protein extractions were different as listed in Appendix). The extract was centrifuged at 8000 rpm for 10 min at 4°C. Supernatant was then filtered through 0.2 μm filter and saved in a new tube.

Concentration of total crude proteins extracted from transgenic *A. thaliana* was measured by the phosphoric acid - Comassie Blue methods using the Bio-Rad Bardford Protein Assay (Bardford, 1976). Thirty millilitres 1X dye was prepared by diluting the 5X dye solution provided in the kit with autoclaved deionized water and filtered through Whatman #1 filter paper. Twenty microlitres BSA standards of concentration 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ ml were added to 980μl diluted dye and the optical densities at 595nm (O.D.595) were measured to establish a standard curve. Twenty microlitres total crude protein sample extracted from transgenic plant

was added to 980 μl diluted dye (the same batch as that used for the standard curve). After recording the sample reading at O.D.595, the protein concentrations of samples was estimated from the standard curve.

2.2.1.6 Generation of cRNA probes for Southern and Northern blot analyses

For generating of cRNA probes for Southern and Northern blot analyses, 2μg of each target cDNA carried in pBluescript II KS (+) was digested with appropriate restriction enzyme at the 5' end of the insert to linearize the plasmid before *in vitro* transcription. The linearized fragment was then purified with Bio-Rad Prep-A-Gene Purification kit. *In vitro* transcription was accomplished with DIG RNA labelling kit (Roche). In the *in vitro* transcription reaction, 1μg linearized and purified pBluescript II KS (+) carrying the target cDNA fragment was added in the reaction as template, T7 or T3 RNA polymerase (basing on the promoter located at the 3' end of the target cDNA) and DIG RNA labelling mix were used to synthesize cRNA labelling with DIG conjugate, in the presence of 4 units/ μl RNase inhibitor (Roche). The 40μl reaction mix was incubated at 37°C for two hours. After the reaction, the product was diluted by 5-folds and the quality was checked by gel electrophoresis of 1μl of the diluted sample.

2.2.1.7 Southern blot analysis

Twenty micrograms genomic DNA sample extracted from transgenic plants was then digested with appropriate restriction enzymes at 37°C overnight. The digested samples were then separated by gel (0.8%) electrophoresis at 70V for approximately 3 hours. The gel was then treated with depurination solution for 5

minutes, denaturation solution for 15 minutes and neutralization solution for 15 minutes. After rinsing in autoclaved deionized water, the gel was equilibrated with 10X SSC solution for 3 minutes before blotting. Blotting was performed for approximately 16 hours in 10X SSC using. The digested DNA fragments were then transferred from the gel to nylon membrane. After UV-crosslinking (total 250J), the membrane was first rinsed in DEPC-treated deionized water and then prehybridized in prehybridization solution at 65°C for 4 hours and hybridized with 25ng/ml cRNA probes in hybridization solution at 65°C for 16 hours. After washing with cold wash solution for 15 minutes twice at room temperature and hot wash solution for 15 minutes twice at 65°C, the membrane was blocked with 1% blocking solution at room temperature for 2 hours and incubated with 1: 10,000 anti-DIG antibody at room temperature for 30 minutes. After washing with maleic acid buffer for 15 minutes twice and equilibrating with detection buffer at room temperature, CSPD substrate was added onto the membrane and X-ray film was allowed to expose for 14 hours.

2.2.1.8 Northern blot analysis

Twenty micrograms of each total RNA sample was run on a denaturing gel. After recording the ethidium bromide stained image of the RNA samples, the RNA was transferred onto a positively charged nylon membrane by capillary action running in the 10X SSC for 16 hours.

Subsequent UV-crosslinking, hybridization and detection steps were the same as described for Southern blot analysis.

2.2.1.9 Expression of lymphocytic choriomeningitis virus nucleoprotein (LCMV-NP) in bacterial system

(i) Expression of LCMV-NP in DE3/pET system

The starter culture of DE3/ pET-LCMV-NP was prepared by inoculating the glycerol stock to 10ml LB broth containing 50mg/ L kanamycin and incubated at 37° C with shaking (200 rpm) (Orbital shaker, Lab. line 4628-1) for 16 hours. Subsequently, 1:100 diluted culture was propagated in 20ml LB broth containing 50mg/ L kanamycin at 37°C until the O.D.600 reached 0.6 to 1.0. Induction of the expression of LCMV-NP was done by adding 1mM IPTG. Culture samples (0.5ml) were harvested at time intervals 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hours after induction. After spinning down the cells at 11, 000g for 2 minutes, the supernatant was removed and the cell pellets were stored at -20°C before further experiments. The cell pellets were subsequently resuspended in 30µl 1X sample loading buffer containing 2% β-mecaptoethanol. After boiling the sample at 100°C for 2 minutes, the samples were analysed by separated onto a 10% SDS-PAGE gel electrophoresing at 20mA constant current. The gel was then stained with Coomassie blue for 30 minutes and destained with fixative solution overnight.

(ii) Purification of His-tag recombinant LCMV-NP from bacterial system

Ten millilitres DE3/ pET-LCMV-NP culture was harvested 4 hours after IPTG induction. After centrifugation at 5000 rpm (Orbital shaker, Lab. line 4628-1) and removal of supernatant, the cell pellet was resuspended in 10ml bacterial cell lysis buffer. The suspension was allowed to stand at room temperature for 30

minutes with occasional shaking. After centrifugation at 8000 rpm for 5 minutes, supernatant was transferred to a new tube and mixed with Prewashed Talon Resin (50μl Talon Resin were first washed with 15μl lysis buffer after removing original bathing solution). The mixture was agitated at room temperature for 10 minutes and centrifuged at 8000 rpm (Orbital shaker, Lab. line 4628-1) for 1 minute. After removing the supernatant, the pellet was washed with 10 ml lysis buffer again. Following centrifugation and removal of supernatant, the prewashed Talon resin and sample bound was transferred to 1.5 ml microcentrifuge tube and resuspended in 1ml lysis buffer. The mixture was centrifuged at 300g for 2 minutes and the pellet was washed with lysis buffer for 3 more times. Washing waste for each round of centrifuge was stored. After removal of the last trace of lysis buffer, 300μl His-tag protein elution buffer was used to elute the His-tag recombinant LCMV-NP protein. Second elution was performed by a further wash with 300μl His-tag protein elution buffer (Porath, *et al.*, 1975).

2.2.1.10 Western blot analysis for LCMV-NP

(i) Purification of anti-LCMV-NP polyclonal antibody obtained from immunized mouse

Serum containing polyclonal antibody against LCMV-NP was obtained from Dr. M. F. Saron (Pasteur Institute, France). The polyclonal antibody was used as the primary antibody in Western blot and protein dot blot experiments to detect recombinant LCMV-NP in transgenic *A. thaliana*. The polyclonal antibody was first purified from the ascites fluid with the MAb Trap GII kit (Pharmacia) before application.

Ten millilitres ascites fluid was filtered through 0.2 μm filter and diluted with 1X binding solution. As the capacity of the commercially available protein column was 5ml per time, 5ml of the total 20ml diluted sample was added to the column with speed of 1 drop per second (the 20% ethanol preservative of the protein column was first removed by washing with 5 ml double distilled water and the column was then equilibrated with 5 ml binding solution in a speed of 1 drop per second). For elution, 3ml elution buffer provided by the commercial kit was passed through the column, also in a speed of 1 drop per second. Elution was collected in a new tube containing 300μl neutralization solution also provided in the commercial kit. The column was then reconditioned with 5 ml binding solution and used to purify another 5ml diluted sample. All steps were repeated until all 20 ml diluted sample was purified.

(ii) Primary antibody and recombinant LCMV-NP validity test

To verify the immunogenic reaction between the purified primary antibody and the recombinant LCMV-NP (produced in bacterial culture), protein dot blot analysis was employed. Serial dilutions of the primary antibody was performed to determine the minimum titer that gave signals to the recombinant LCMV-NP protein.

Six drops (1μl each) recombinant LCMV-NP protein generated from bacterial culture were dotted onto a methanol and 1 X PBS buffer pretreated PVDF membrane. The dots were air-dried and the membrane was cut into six pieces, each contained a dot of the target protein. The membrane pieces were blocked with Aurora^{TM} blocking solution at room temperature for 45 minutes with gentle shaking before reacted with the serial diluted primary antibody (1:50, 1:500, 1:2500, 1:5000, 1: 7500 and 1:10000) in blocking solution, at room temperature for 45 minutes with

gentle shaking. The primary antibody used was pretreated by preabsorbing with total protein extracted from transgenic *A. thaliana* containing the empty T-DNA from the binary vector V7. The membranes were washed with Aurora™ blocking solution at room temperature for 5 minutes twice. After the washing steps, the membranes were hybridized with secondary antibody (1:5000) in blocking solution at room temperature for 45 minutes with gentle shaking. Before proceeding to the detection step, the membranes were washed with blocking solution at room temperature for 5 minutes three times. The membranes were then incubated in 1X assay buffer for 2 minutes twice. After addition of chemiluminescent Starlight™ substrate solution containing 5% Opti-Membrane reagent, the membranes were expose to X-ray film for 30 minutes.

(iii) Westing blotting

After testing the validity of using the recombinant LCMV-NP as positive control and determining the titer of primary antibody required, Western blot was performed to test the presence of recombinant LCMV-NP in transgenic *A. thaliana.* Approximately 7.5μg total crude protein extracted from each transgenic lines were used to run onto a 10% SDS-PAGE gel electrophoresis. Protein samples from transgenic *A. thaliana* containing the empty T-DNA and wild-type (Col-0) were used as negative controls. The proteins separated on the gel were then transferred to the 100% Methanol and transfer buffer pre-treated PVDF membrane (the membrane was first bathed in absolute methanol for 20 minutes and protein transfer buffer for 15 minutes) using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell. The transfer was performed at 15V for 30 minutes. The membrane were then blocked with Aurora™ blocking solution at room temperature for 45 minutes with gentle

shaking and reacted in the blocking solution with primary antibody (in a dilution of 1:50) at room temperature for 45 minutes with gentle shaking (the primary antibody was pretreated as described in the last section. The membrane was washed with blocking solution at room temperature for 5 minutes twice. After the washing steps, the membrane was further reacted with the secondary antibody in blocking solution in ratio of 1:5000 at room temperature for 45 minutes with gentle shaking. The detection procedures were performed as described in the last section.

2.2.1.11 Protein dot blot detection of recombinant LCMV-NP generated from transgenic plants

PVDF membrane was first treated with 100% methanol for 20 minutes and 1X PBS buffer for 15 minutes. The membrane was then put in a dot blot microfiltration apparatus (Bio-Rad). Five hundred microlitres 1X PBS was applied to each wells with vacuum drawing before 9, 18, 27, 36, 45 and 54μg protein extracted from transgenic plants were added to the washed wells. After all samples had passed through the membrane by vacuum, the wells were further washed with 500μl 1X PBS twice. The membrane was then blocked with blocking solution at room temperature for 45 minutes with gentle shaking and all the subsequent steps were performed as described in the previous section.

2.2.1.12 PCR techniques

For PCR-aided sequencing, 0.5-1.0μg DNA sample was used as the template. In a 20μl reaction mixture, 0.8pmole primer and 8μl terminator ready reaction mix were included. The PCR cycle profile was as follows: 94°C for 5 minutes before 25

cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. For generating DNA fragment for subcloning and PCR screening, the PCR reaction mixture contained 0.5 μM of each primer, 1X PCR buffer with 1.5mM $MgCl_2$, 0.2mM dNTPs and 0.5 Unit Taq DNA polymerase were reacted in a final volume of 25μl. The PCR cycle profile was as follows: 94°C for 5 minutes before 25 cycles of 94°C for 30 seconds, appropriate annealing temperature for 30 seconds, 72°C for 1 minutes and the reaction was terminated after an addition 10-minute extension step after all cycles were completed (the annealing temperature for each primer was listed in Table 13).

Table 13 Annealing temperatures of primers used in PCR reactions.

Primer name	Use for	Annealing temperature
35S promoter sequencing primer	Sequencing	50.0°C
T3 primer	Sequencing	50.0°C
T7 primer	Sequencing	50.0°C
HMOL 538 & 539	PCR screening of LCMV-NP clones	50.0°C
HMOL 540 & 541	PCR screening of LCMV-NP clones	50.0°C
HMOL 643 & 646	Amplification of LCMV-NP with *BamHI* at 5' end and *Sac*I at 3'end	51.6°C
HMOL 703 & 704	PCR screening for GUS transgene	50.0°C
HMOL 705 & 706	PCR screening for *virA* gene	53.0°C

2.2.1.13 Sequencing

PCR-aided DNA sequencing was performed by using the ABI prism dRhodamine terminator cycle sequencing ready reaction kit (Applied Biosystems) to make labeled single strand DNA (as described in 2.2.1.12). The PCR reaction

product was added with 2μl 3M sodium acetate (pH5.2) and 50μl 95% ethanol. The mixture was kept on ice for 30 minutes and centrifuged at 14000g for 30 minutes. The DNA pellet was washed in 70% ethanol after removal of supernatant. The washed and air-dried pellet was then resuspended in 15μl Template Suppression Reagent. The sample was then applied to the Genetic Analyzer ABI prism 310 for analysis.

2.2.2 Plant tissue culture and transformation

2.2.2.1 *Arabidopsis thaliana*

Surface sterilization of seeds

Seeds of *A thaliana* were first surface sterilized in 100% Clorox for 3 minutes with vigorous shaking. Before rinsing with autoclaved double distilled water for 3 times to remove Clorox. The surface sterilized seeds were either individually placed and aligned onto MS agar square plates for growth or spread onto MS agar plate with 50mg/ L kanamycin for screening of transformants. After keeping in dark at 4°C for 2 days, the seeds were grown in growth chamber at 22°C with 70% relative humidity under the light/ dark cycle of 16 hours light and 8 hours dark.

Transformation of *A. thaliana*

Four pots each containing 4 *A. thaliana* plants (6-12 inches tall), were used for transformation. *A. thaliana* grown to flowering stage was ready for vacuum-assisted *Agrobacterium*-mediated transformation. All sliques were removed

and the rosette leaves and soil were covered with parafilm. The plants were inverted and immersed into the agrobacterial suspension. Vacuum was applied at 400 mm Hg was drawn for 10 minutes after bubbles were seen. The plants were then allowed to recover and shed seeds. Seeds of the transformed plants were harvested separately after the plants were allowed to dry (by cutting off water supply) for 4-6 weeks.

2.2.2.2 Soybean

Surface-sterilization of soybean seeds

The seeds were first rinsed with 75% ethanol and then washed with 25% clorox plus a few drops of Tween-20 for 30 minutes with occasional shaking. The seeds were then rinsed with autoclaved double distilled water for five times and imbibed in autoclaved double distilled water for 3 hours before sowing in half MS agar. The seeds were grown in growth chambers with 70% relative humidity and 16 hours light and 8 hours dark daylight cycle at 26°C.

Establishment of soybean regeneration system

As *Agrobacterium*-mediated transformation of soybean cotyledonary node was adopted to construct transgenic soybean, the regeneration platform for soybean cotyledonary node explants was a prerequisite (Trick, *et al.*, 1997).

The germinated soybean seeds were used to prepare the cotyledonary explants. After complete stripping of the seed coat and removal of the radicle 2 mm below the junction of the seedling cotyledon, each seed was bisected to yield two

identical explants. About one-fourth of each cotyledon tip was cut off. Explants were macerated through the meristem and cotyledonary nodes to disrupt primary shoot morphogenesis and provide wound sites for regeneration and *Agrobacterium* infection. The cotyledonary node explants prepared were soaked in explant preparatory medium for 30 minutes before transferred to regeneration medium for regeneration or to *Agrobacterium* culture solution for transformation.

The cotyledonary explants were then plated in differentiation medium with adaxial side up. The explants were cultivated in growth chamber with 70% relative humidity and 16 hours light and 8 hours dark daylight cycle at 26 °C.

After the differentiation of shoot and stem, the plantlets were transferred to stimulating medium. Under low BA to IBA ratio, roots were induced. After the formation of the primary root, the plantlet was transferred to half MS medium lacking any phytohormones. Cultivation condition was the same as described above.

Establishment of transformation system

For *Agrobacterium*-mediated soybean transformation, *Agrobacteria* containing clones of interests were cultivated in YEP medium supplemented with 25 mg/ L rifampicin, 50mg/ L gentamycin and 50mg/ L kanamycin (for selection of V7, W104 and pBI121) at 28°C with shaking (250 rpm) for 2-3 days. The culture was harvested and resuspended with resuspension medium to attain $O.D._{600}$ of about 1.0.

The explants preparation for transformation was the same as described for explant regeneration. After soaking the explants in explant stimulating medium for

approximately 30 minutes, they were transferred to the *Agrobacterium* culture and co-cultivated for 1 hour. After co-cultivation, the explants were placed to solid co-cultivation medium with adaxial side up and incubated in dark for 72 hours.

After dark incubation, the explants were first washed with autoclaved distilled water for three times before rinsing with explant washing medium once and soaking in explant washing medium for 1.5 hours. The explants were then inserted into the differentiation medium with adaxial side up and cultivated as described above. Kanamycin, if involved, was added to the final concentration of 50mg/ L (Trick, *et al.*, 1997).

After shoot differentiation, the explants were subcultured in root stimulating medium and MS medium as mentioned in 2.2.2.2.2.

GUS staining of GUS gene transformed explants

Plant tissues such as regenerated buds or leaves, were firstly rinsed with double distilled water and then soaked in GUS staining reagent at 37°C for 12 hours. The samples were then treated with 70% ethanol for 24 hours to eliminate chlorophyll and enhance the colorimetric detection of GUS activities.

2.2.3 *In vitro* transcription and translation of target genes in rabbit reticulocyte and wheat germ systems

2.2.3.1 *In vitro* transcription of target genes

In vitro transcription was performed with Ribomix large scale RNA production systems-T7 and SP6 (Promega). Target genes carried in pGEM-3Zf(+) vector were firstly digested with appropriate restriction enzymes (LCMV-NP and GHI: *Xba*I; GHII: *Hind*III) at 3' ends before the *in vitro* transcription process. The linearized plasmids with target genes were then purified with PCI extraction and ethanol precipitation.

Based on the orientation of the inserts, mRNA transcript of the target genes were generated different promoters. The pGEM-3Zf(+)-GHI and pGEM-3Zf(+)-LCMV-NP used SP6 promoter, while pGEM-GHII used T7 promoter. The transcription reaction mixes included 7.5 μg linearized DNA, 25mM rNTPs, 1X transcription buffer and 1X enzyme mix. The transcription buffer, rNTPs and enzyme mix were provided in the commercial kits (Promega). The reaction for generating luciferase positive control (control in the kit) was also performed. The reaction mixes were incubated at 37 ° C for 4 hours. After transcription, denaturing gel electrophoresis was performed to check the qualities and quantities of transcripts.

2.2.3.2 *In vitro* translation with rabbit reticulocyte lysate and wheat germ extract

***In vitro* translation with rabbit reticulocyte lysate**

Ten micrograms RNA sample produced in 2.2.3.1 and 2μl Transcend™ biotin-lysyl-tRNA were added to a 50μl reaction mix which included 35μl rabbit reticulocyte lysate, 0.5μl 1mM amino acid mixture (without leucine), 0.5μl 1mM amino acid mixture (without methionine), and 40 units RNasin® ribonuclease inhibitor. Reagents except RNasin® ribonuclease inhibitor were provided in the rabbit reticulocyte lysate and transcend non-radioactive (chemiluminescent) translation detection systems (Promega). To check the efficiency of the translation system and detect non-specific translated products, positive control (luciferase) and negative control (no RNA templates) were also tested. One microgram positive RNA control (luciferase) provided in the commercial kit was added in the positive control reaction solution and only 1 μl Transcend™ tRNA was added in both positive and negative control reactions. The reactions were incubated at 30°C for 1 hour.

***In vitro* translation with wheat germ extract**

About 10μg RNA samples produced in 2.2.3.1 were added to a 50μl reaction solution which included 25μl wheat germ extract, 2 μl 1M potassium acetate and 2μl Transcend™ biotin-lysyl-tRNA, 2μl 1mM amino acid mixture (without leucine), 2μl 1mM amino acid mixture (without methionine) and 40 units RNasin® ribonuclease inhibitor. Reagents except RNasin® ribonuclease inhibitor were provided from the wheat germ extract and transcend non-radioactive (chemiluminescent) translation detection systems (Promega). Besides, reactions using 1μg Brome Mosaic Viurs

(BMV) RNA positive control provided by the kit and negative control (no RNA templates) were also performed. All reactions were incubated at 25°C for 1.5 hours.

Detection of target biotinylated labeled proteins generated by rabbit reticulocyte lysate and wheat germ extract

Fifteen percent SDS-PAGE gel electrophoresis was used to detect the target GHI and GHII protein with size about 23kDa, while 10% SDS-PAGE gel was used to resolve the LCMV-NP with size around 61kDa. After gel electrophoresis, electroblotting was performed to transfer the proteins to PVDF sequencing membrane with the aid of the Trans-Blot® SD Semi-Dry Electorphoretic Transfer Cell (15V for 30min). After blocking of the membrane with Tris-buffered saline (TBS) (pH7.5) for 1 hour, hybridization of Strept.-HRP (1:5000) in TBS was performed for another hour. The membrane was washed three times first with TBS with 0.5% Tween 20 (TBST) and then placing in TBS. Detection was accomplished by adding 2ml Substrate A & B (1:1) to the membrane and exposing the X-ray film overnight.

Chapter 3 Results

3.1 Expression of Lymphocytic Choriomeningitis Virus Nucleoprotein (LCMV-NP) and Goldfish Growth Hormones I and II (GHI and GHII) in Transgenic *Arabidopsis thaliana*

3.1.1 Expression of LCMV-NP in transgenic *Arabidopsis thaliana*

3.1.1.1 Subcloning of LCMV-NP cDNA into the binary vector system W104

The full-length cDNA clone of LCMV-NP was obtained from Dr. M. F. Saron, our collaborator at Pasteur Institute, France. The cDNA was originally inserted into a pUC plasmid and the flanking restriction sites were shown in Fig. 1 (Saron, M. F., personal communication).

To perform *Agrobacterium*-mediated transformation, the LCMV-NP cDNA was first subcloned into the binary vector V7 (Brears, *et al.*, 1993). The cloning sites and important features of V7 were shown in Fig. 2.

The plasmid DNA for pUC-LCMV-NP and V7 were prepared as described in Chapter 2. The pUC-LCMV-NP was digested with *EcoR*I and *Bbu*I, and V7 was digested with *Xho*I and *Xba*I. The target LCMV-NP cDNA fragment and the digested V7 plasmid were then purified by agarose gel electrophoresis (Fig. 3).

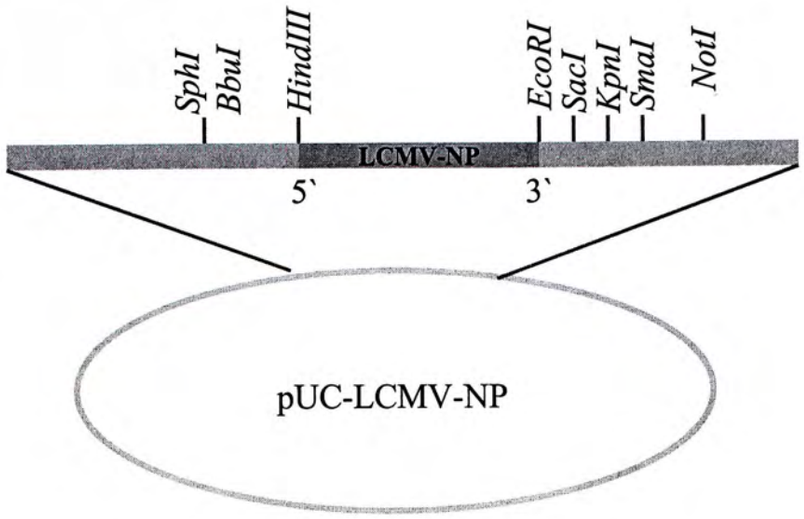


Fig. 1. The schematic diagram of the full-length LCMV-NP cDNA fragment cloned in a pUC based vector.

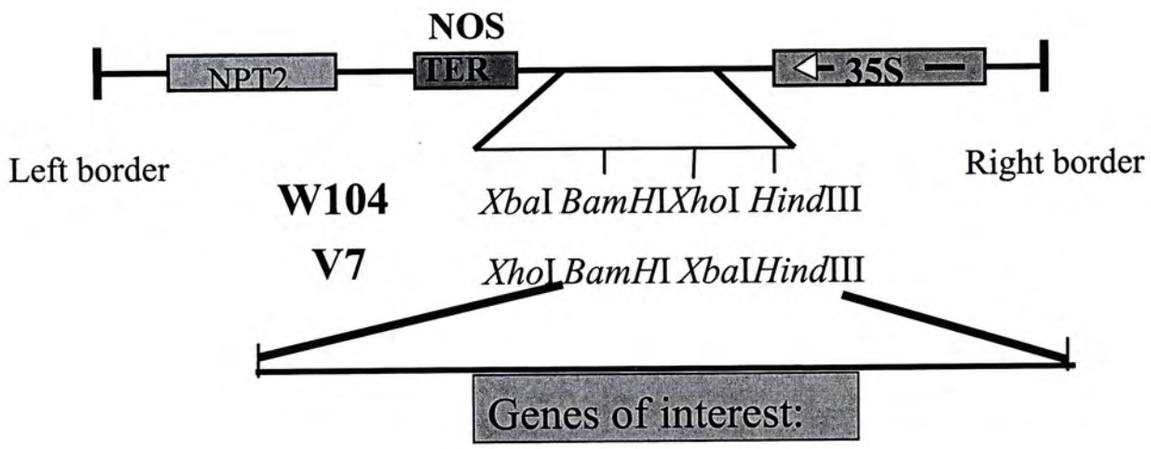
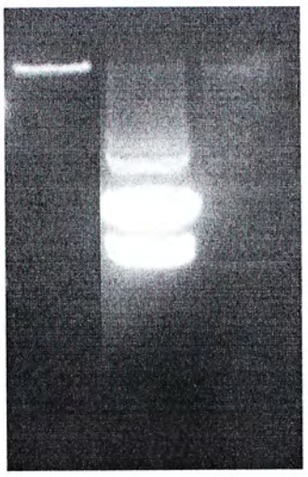


Fig. 2. Cloning sites and important features of the V7 and W104 vectors.

(a)



(b)

Fig. 3. The gel photo showing digested and purified products of pUC-LCMV-NP and V7. (a) Two micrograms of pUC-LCMV-NP digested with *Bbu*I and *EcoR*I enzymes and 1µg of V7 digested with *Xho*l and *Xba*I for 6 hours at 37°C and subsequently separated on 0.8% agarose gel. (b) Purified DNA fragments from (a) were re-analyzed on 0.8% agarose gel.

*Xba*I-*Bbu*I and *EcoR*I-*Xho*I adapters (see below and also the Materials and Methods Section) were ligated to the *Bbu*I and *EcoR*I ends, respectively, of the purified LCMV-NP cDNA fragment.

*Xba*I-*Bbu*I adapter:

*Xba*I *Hind*III *Not*I *Bbu*l

5'CTAGAAGCTTGCGGCCGCCATG 3' HMOL 535

3'TTCGAACGCCGGCG 5' HMOL 543

*EcoR*I-*Xho*I adapter:

*Xho*I *Not*I *EcoR*I

5'TCGAGCGGCCGC 3' HMOL 533

3'CGCCGGCGTTAA 5' HMOL 532

*Xba*I and *Xho*I digested V7 plasmid was added to the same ligation mixture. The final ligated products were transformed into DH5α and successful transformants were selected on LB agar plate containing 50mg/ L kanamycin. Plasmids from a total of six putative candidates were prepared and their insert sizes were confirmed by digestion with *Xba*I and *Xho*I (Fig. 4)

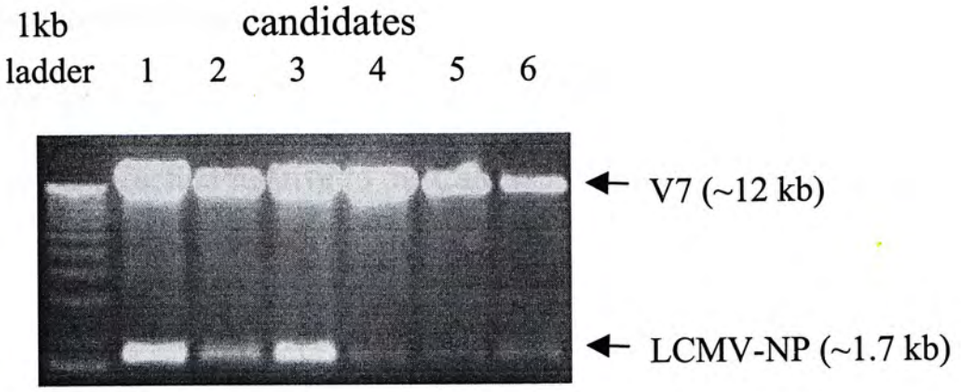


Fig. 4. Result of *Xho*I and *Xba*I digestion of the six putative V7-LCMV-NP candidates. The 12kb and 1.7kb bands were the sizes as the V7 vector and LCMV-NP full-length cDNA, respectively. All the six candidates showed positive result.

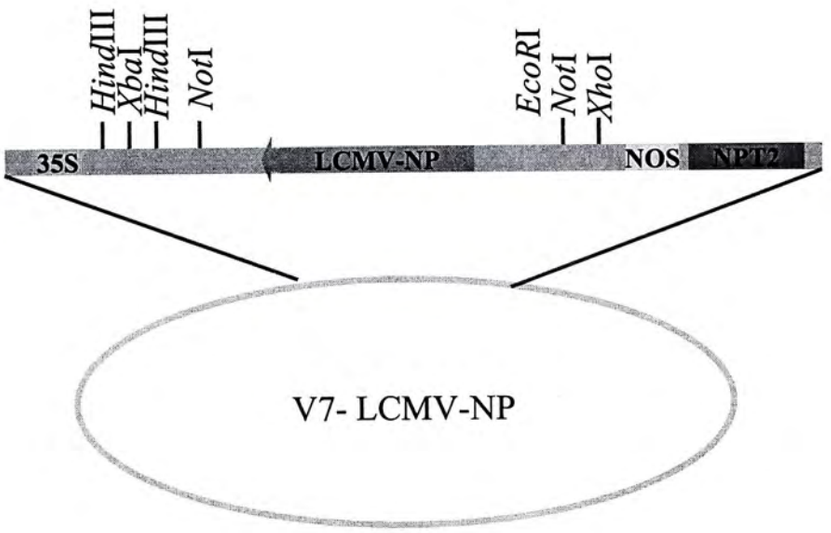


Fig. 5. The schematic diagram of V7-LCMV-NP. LCMV-NP cDNA was inserted in an anti-sense positive relative to the CaMV 35S promoter.

Partial DNA sequencing of all six candidates were determined using the 35S primer (an internal sequence of the CaMV 35S promoter upstream of the cloning site). Results showed that all clones contained LCMV-NP cDNA fragment inserted in an anti-sense direction relative to the CaMV 35S promoter (Fig. 5).

To circumvent this problem, the binary vector W104 (Brears, *et al.*, 1993) was employed. W104 is identical to V7 except the *Xba*I and *Xho*I sites were arranged in at opposite orientation (Fig. 2).

Both V7-LCMV-NP and the W104 vector were digested with *Xba*I and *Xho*I. After gel purification of the LCMV-NP fragment and the digested W104, sticky end ligation was performed. Eight transformants were obtained. PCR screening verified that all of them possessed the LCMV-NP fragment (Fig. 6). The final construct of W104-LCMV-NP was shown in Fig. 7.

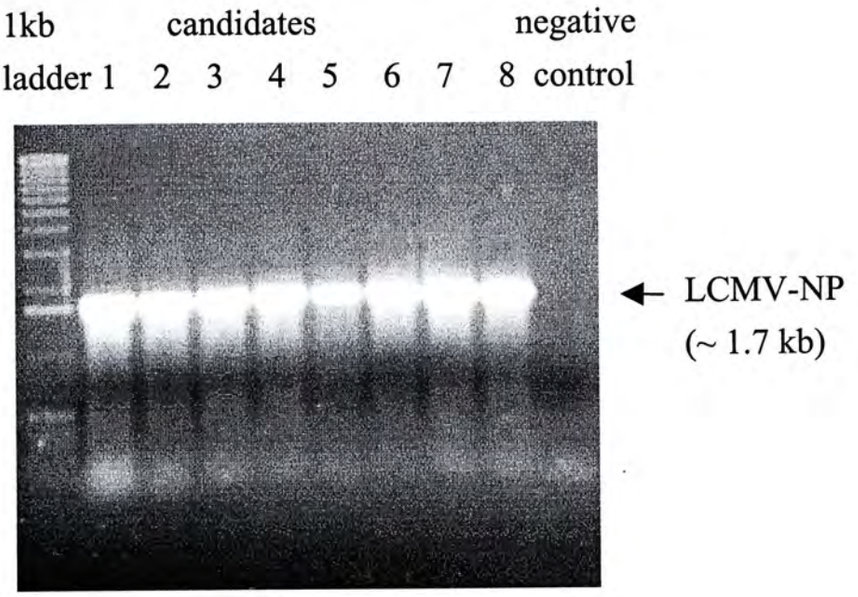


Fig. 6. PCR screening of W104-LCMV-NP putative candidates. PCR screening was performed using oligos HMOL 538 and HMOL 541 as described in the Materials and Methods Section. All candidates except the negative control (W104 plasmid without insert) gave an amplified band of about 1.7kb, the expected size of the LCMV-NP cDNA fragment.

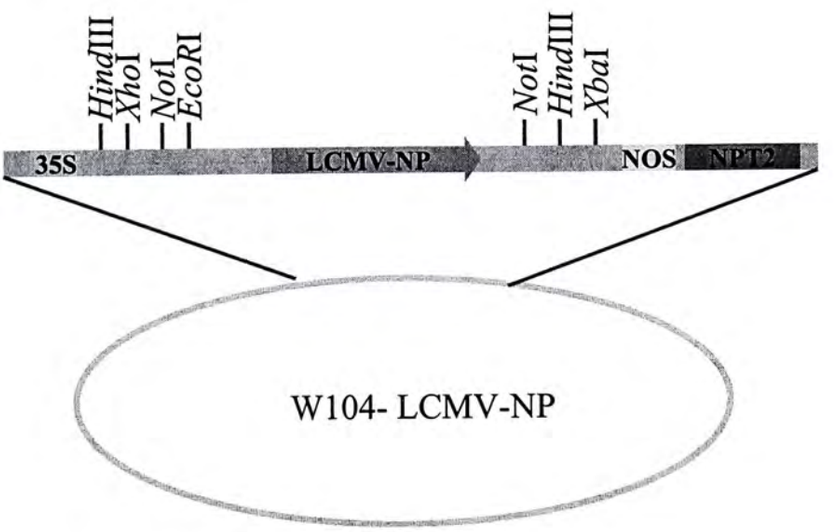


Fig. 7. The schematic diagram of W104-LCMV-NP.

The orientation of all candidates was in the same orientation relative to the CaMV 35S promoter, as confirmed by sequencing experiment using 35S primer. This result is expected, since directional cloning using double digestion was performed.

3.1.1.2 Transformation of W104-LCMV-NP into the *Agrobacterium* GV3101/ pMP90

To perform *Agrobacterium*-mediated transformation, the recombinant plasmid W104-LCMV-NP generated was transformed into the *Agrobacterium tumefaciens* host. GV3101/ pMP90, a commonly used strain for *Agrobacterium*-mediated plant transformation especially when vacuum infiltration was employed. Using electroporation (see the Materials and Methods Section), the W104-LCMV-NP plasmid was transformed into GV3101/ pMP90. Successful transformation events were screened by PCR using oligonucleotides HMOL 538 and HMOL 541 (Fig. 8).

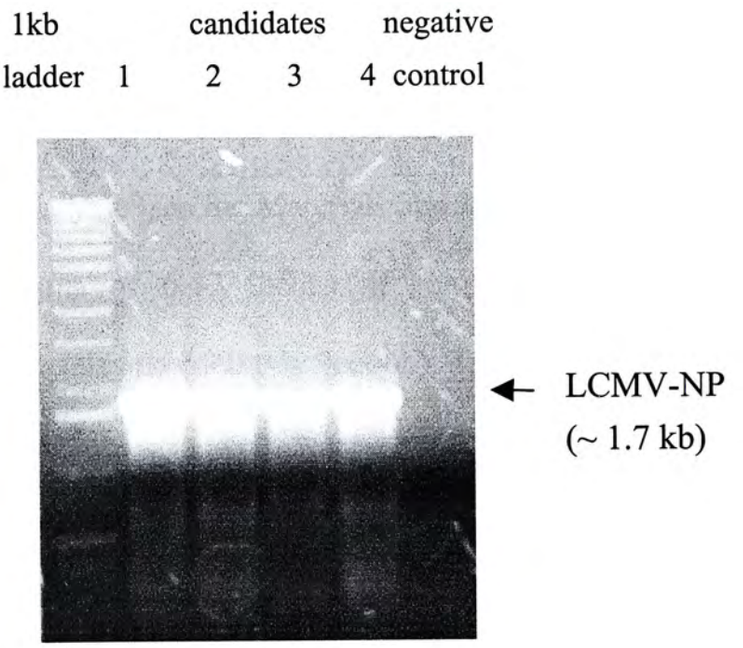


Fig. 8. PCR screening of GV3101/ pMP90 transformants containing the plasmid W104-LCMV-NP. All tested candidates gave a band of size around 1.7kb. Empty W104 plasmid was used as a negative control.

3.1.1.3 Transformation of LCMV-NP cDNA into *Arabidopsis thaliana*

Arabidopsis thaliana ecotype Col-0 was used as the plant host for transformation. Plants were grown in an environmentally controlled growth chamber under a regular day-light cycle (see the Materials and Methods Section). A total of 24 flower bud-bearing plants of 6-12 inches in height were chosen for vacuum infiltration (see the Materials and Methods Section). Treated plants were rescued and allowed to grow and shed seeds. T_1 seeds were screened on MS agar plate containing 50mg/ L kanamycin. After germination and development of cotyledons, successful transformants continued to develop into green seedlings with true leaves while untransformed individuals ceased to grow and turned yellow (Fig. 9).

A total of nine transformants were obtained and designated as HML 603 A-1, HML 603 A-3, HML 603 C-2, HML 603 D-1-1, HML 603 D-1-2, HML 603 D-1-3, HML 603 D-1-4, HML 604 C-1-1 and HML 604 C-1-2. The nine transformants were individually cultivated in growth chamber under the conditions described in the Materials and Methods Section. Their seeds were harvested and more than 100 T_2 seeds of each transformants were screened on MS agar plate containing 50 mg/ L kanamycin. The number of green transformants versus yellow seedlings was recorded and chi-square test was performed (Table 14). Results of Chi-square test suggested that each of the transformed lines HML 603 C-2, HML 603 D-1-2, HML 603 D-1-3 and HML 604 C-1-1 contained a single insertion locus.

Table 14 Chi-square test for a 3:1 green: yellow ratio of selfed progenies of successful LCMV-NP transformants[(a)].

Lines	# Green	# Yellow	X^2 Value	Conclusion
HM603 A-1	44	1	12.42	rejected
HM603 A-3	86	0	28.67	rejected
HM603 C-2	57	19	0.00	single insertion locus
HM603 D-1-1	101	16	8.00	rejected
HM603 D-1-2	58	16	0.45	single insertion locus
HM603 D-1-3	50	19	0.24	single insertion locus
HM603 D-1-4	69	3	16.66	rejected
HM604 C-1-1	71	16	2.03	single insertion locus
HM604 C-1-2	75	9	9.14	rejected

[a]Significance level was set of 0.05 and the critical value was calculated as 3.841.

Kanamycin resistant progenies of transformed lines were selfed and allowed to shed seeds. About 100 T_3 seeds of each line were then screened on MS agar plates containing 50mg/ L kanamycin. Homozygous lines were identified in case all T_3 progenies were kanamycin resistant.

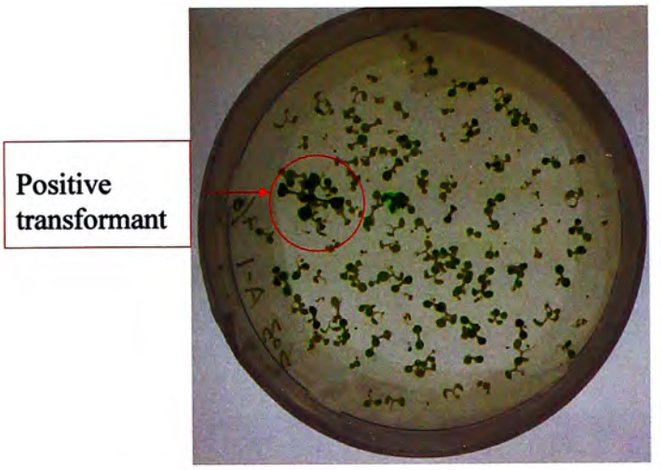


Fig. 9. Screening of LCMV-NP transgenic *A. thaliana*. Positive candidates appeared as green seedlings with true leaves. Untransformed individuals ceased growth and appeared yellow. A total of 9 positive candidates were found out of 24 parent plants used for transformation. They were designated as HML603 A-1, A-3, C-2, D-1 (1), D-1 (2), D-1 (3), D-1 (4), HML604 C-1(1) and C-1(2). HML 603 A-1 was shown as the figure.

The homozygous lines obtained included HML603C-2-G, HML603D-1-2-E, HML603D-1-2-G, HML603D-1-2-I, HML603D-1-4-M, HML603D-1-4-N, HML604C-1-1-A, HML604C-1-1-B, HML604C-1-1-G, HML604C-1-1-K and HML604C-1-1-O. The eleven homozygous lines were from 4 independent origins (Group 1: HML603C-2-G; Group 2: HML603D-1-2-E, HML603D-1-2-G and HML603D-1-2-I; Group 3: HML603D-1-4-M and HML603D-1-4-N; Group 4: HML604C-1-1-B, HML604C-1-1-G, HML604C-1-1-K and HML604C-1-1-O).

3.1.1.4 Southern blot and Northern blot analyses of transgenic plant containing the LCMV-NP cDNA

T_3 plants from all nine primary transformants were grown for 4 weeks. DNA (for four transformants with single insertion locus) and RNA (for all nine transformed lines) samples were extracted as described in the Materials and Methods Section.

To perform Southern blot and Northern blot analyses, DIG-labeled cRNA probes of LCMV-NP were prepared (see the Materials and Methods Section). *Xho*I and *Xba*I fragment of LCMV-NP cDNA was cleaved from W104-LCMV-NP by restriction enzymes. The pBluescript KSII (+) plasmid cut with the same enzymes were used to clone the LCMV-NP cDNA fragment (Fig. 10).

After sticky end ligation and transformation into the bacterial host DH5α, successful constructs were screened by PCR (Fig. 11). The schematic diagram of pKS-LCMV-NP was shown in Fig. 12.

DIG-labeled cRNA of LCMV-NP was generated with T7 RNA polymerase after linearization of the plasmid at *Xho*I site (see the Materials and Methods Section). Complementary RNA synthesis was confirmed by running the *in vitro* transcripted products on an agarose gel.

In Southern blot experiment, the genomic DNA samples extracted were first digested with *Xba*I before separated on 1% agarose gel by electrophoresis. The result of Southern blot shown in Fig. 13 confirmed that the LCMV-NP cDNA fragment was inserted into the plant genome. A single band of slightly different sizes were observed in four independent lines, confirming that a single copy of LCMV-NP cDNA was inserted into an independent locus in each line.

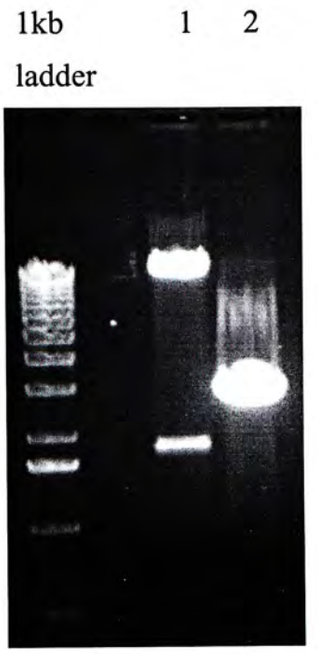


Fig. 10. Digestion of W104-LCMV-NP and pBluescript II KS (+) with *Xho*I and *Xba*I. Lane 1: W104-LCMV-NP; lane 2: pBluescript KS II (+).

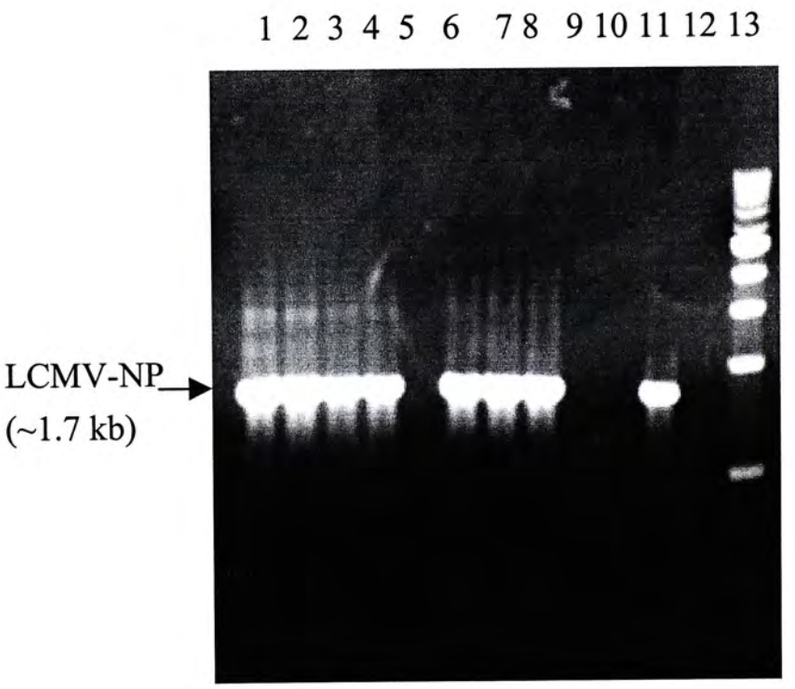


Fig. 11. PCR screening of DH5α/pBluescript II KS (+)-LCMV-NP. PCR screening was performed using oligos HMOL 538 and 541 primers as described in the Materials and Methods Section. Lanes 1-8: putative candidates; lanes 9 and 12: blanks; lane 10: positive control; lane 11: negative controls and lane 13: 1kb ladder. Lanes 1-4, 6-8 gave positive results.

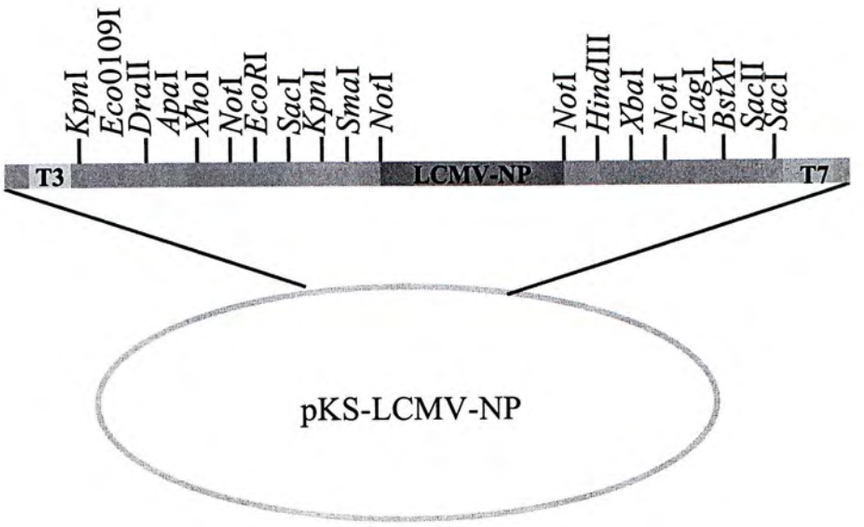


Fig. 12. The schematic diagram of pKS-LCMV-NP.

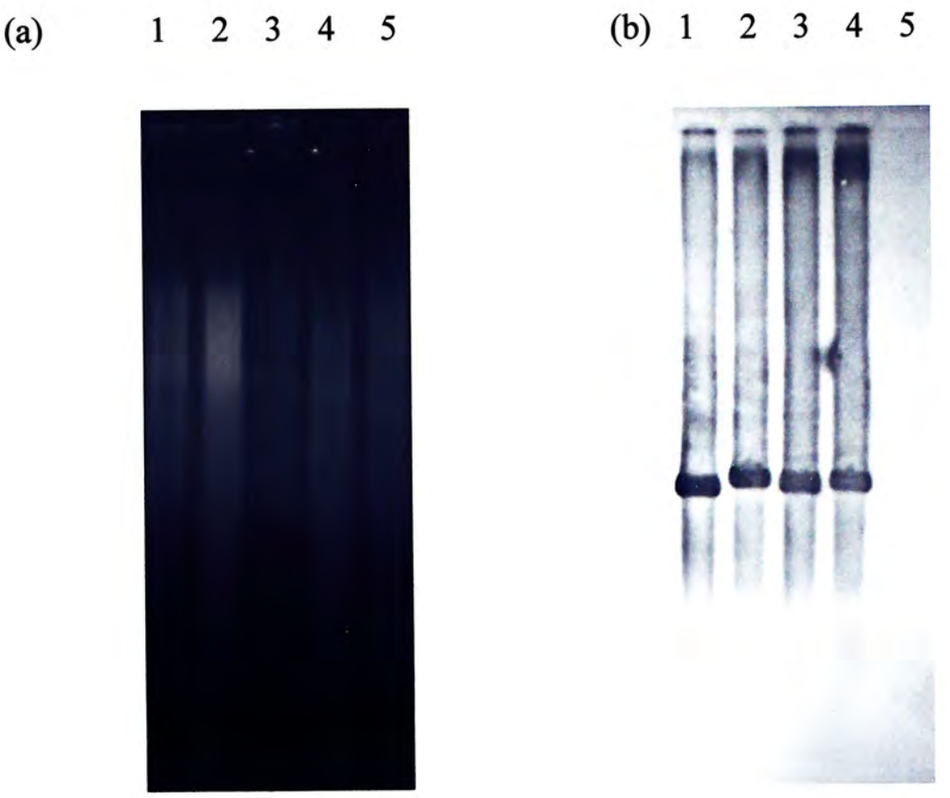


Fig. 13. Southern blot analysis of LCMV-NP transgenic plants. (a) Ethidium bromide staining of genomic DNA digested with *Xba*I. (b) Autoradiograph of Southern blot. Lane 1: HML603 C-2; lane 2: HML603 D-1-2; lane: HML603 D-1-3; lane 4: HML604 C-1-1 and lane 5: *A. thaliana* transformant with T-DNA from an empty V7 vector.

For Northern blot analysis, total RNA from LCMV-NP transformants at both heterozygous stage (all nine transformed lines) and homozygous stage (the eleven transgenic lines originated from the four independent groups) was extracted and separated on an 1% denaturing gel as described in the Materials and Methods section. The result of Northern blot analysis was shown in Fig. 14 and Fig. 15. All the nine heterozygous and eleven homozygous transformants gave positive signal when the LCMV-NP cRNA probe was used. No signal was detected in the two negative controls: RNA samples extracted from *A. thaliana* transformed with the T-DNA from empty V7 vector or from wild-type Col-0.

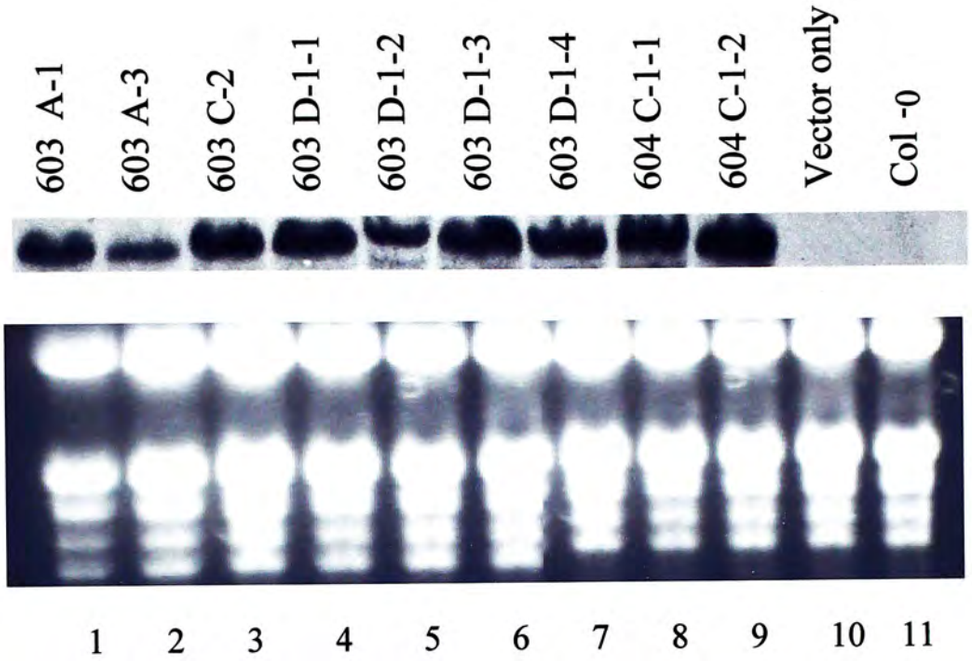


Fig. 14. Northern blot analysis of LCMV-NP transgenic *A. thaliana* at heterozygous stage. The transgene expression was confirmed in all the nine transformants (lanes 1-9) when compared to the two negative controls (lanes 10 & 11).

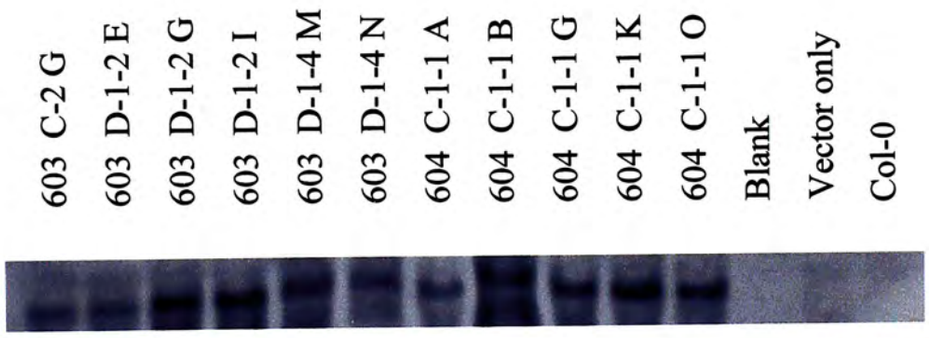


Fig. 15. Northern blot analysis of the LCMV-NP transgenic *A. thaliana* at homozygous stage. Four LCMV-NP transgenic *A. thaliana* each carried a single insert was followed to the homozygous stage. All of them gave positive results to the LCMV-NP cRNA probes, when compared with the two negative controls.

3.1.1.5 Production of recombinant LCMV-NP protein in DE3 cells

To provide a positive control for the Western blot and protein dot blot analyses of LCMV-NP transgenic plants, recombinant LCMV-NP protein was generated independently via a bacterial system. Bacterial expression vector system, pET-30a (+) was employed. LCMV-NP full-length cDNA fragment was subcloned in frame with the His-tag coding sequence. The LCMV-NP full-length cDNA fragment with *BamH*I and *SacI* cutting sites just before and after the start and stop codons respectively was generated by PCR amplification using oligos HMOL 643 and HMOL 646 (Fig. 16) as described in the Materials and Methods Section. The pET-30a (+) vector digested with *BamH*I and *Sac*I was then ligated with the PCR fragment via the compatible ends. After transformation and selection on LB agar plate containing 50mg/ L kanamycin, two candidates were picked and screened with oligos HMOL 540 and HMOL 541 (Fig. 17).

Since the inserted LCMV-NP cDNA fragment in pET-30a was a PCR product, it was important to check for PCR-prone error. The pET-30a (+) - LCMV-NP recombinatn plasmid was digested with *Xho*I and *Xba*I to release the LCMV-NP cDNA fragment. The cDNA fragment was then ligated to the *Xho*I and *Xba*I restricted pBluescript II KS (+) plasmid vector. The ligation mixture was subsequently used to transform DH5α competent cells. Transformed cells were spread on LB agar plate containing 100mg/ L ampicillin and incubated at 37°C overnight. Eight colonies were picked and screened by PCR using oligos HMOL 540 and HMOL 541 as primers (Fig. 18). Five out of the eight candidates gave positive results.

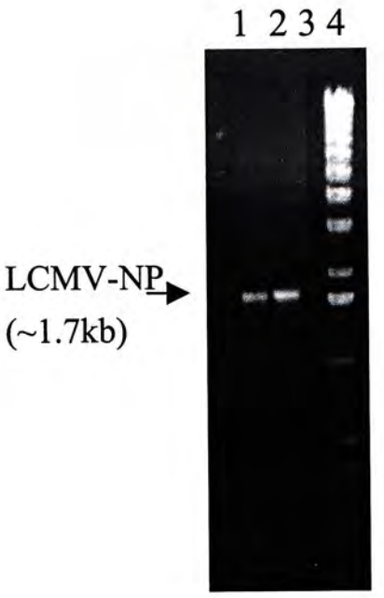


Fig. 16. PCR amplification of full-length LCMV-NP fragment with *BamH*I and *Sac*I ends. LCMV-NP cDNA fragment was amplified from pUC-LCMV-NP recombinant plasmid using HMOL 643 and HMOL 646 as primers. Lanes 1 and 2: PCR samples amplified from different reaction tubes using the same reaction mix components; lane 3: blank; and lane 4: 1kb ladder (Gibco).

Fig. 17. PCR screening of DH5α/ pET-30a (+) - LCMV-NP. Positive candidates were screened by PCR method using oligos HMOL 540 and 541 as primers. Lanes 1 and 2: two candidates. Lane 3: negative control with empty pET-30a(+); lane 4: positive control with pUC-LCMV-NP; and lane 5: 1kb ladder (Gibco). The two samples gave bands of expected size (about 470bp).

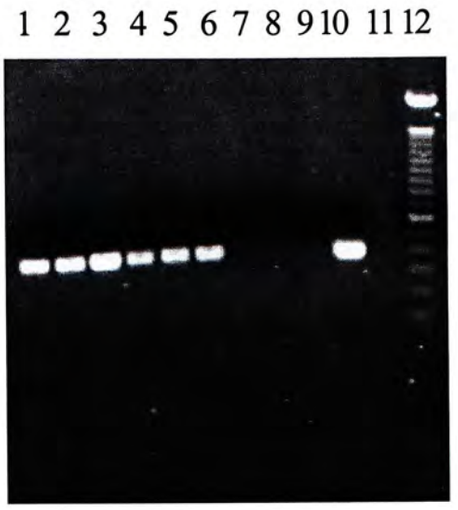


Fig. 18. PCR screening of DH5α/ pKS-pET-LCMV-NP candidates. Positive candidates were screened by PCR method using oligos with HMOL 540 and HMOL 541 as primers. Lanes 1-8: candidates; lane 9: negative control with empty pBluescript KS II (+); lane 10: positive control with pUC-LCMV-NP; lane 11: blank; and lane 12: 100bp ladder (Gibco). The first six candidates gave bands of expected size (about 470bp).

To verify that the LCMV-NP coding region (generated by PCR) in the recombinant plasmid pET-30a(+)-LCMV-NP was free from PCR-brone error, two measures were taken. DNA sequencing was performed from both ends using T3 or T7 primers. No errors were found from about 600bp from each end. Furthermore, the middle part of LCMV-NP cDNA was cut out from pET-30a(+)-LCMV-NP using *Dra*I and replaced with a homologous *Dra*I fragment released from pBluescript KSII (+)-LCMV-NP (Fig. 19). After transformation into DH5α and incubated on LB agar plate containing 50mg/ L kanamycin at 37°C overnight, four colonies were picked and inoculated into 5 ml LB broth containing 50mg/ L kanamycin. After cultivation overnight, plasmids from the four candidates were extracted using Wizard Plus Minipreps DNA Purification kit. The samples were digested with *Bgl*II to check the presence of insert and the orientation. One positive candidate was obtained as indicated by giving restriction bands of 1196bp, 410bp and 5595bp. The other three candidates contained no insert gave bands of 749bp and 5595bp (Fig. 20).

The pET-30a (+) - LCMV-NP construct was then transformed into DE3 competent cells (Phillips, *et al.*, 1984; Wood, *et al.*, 1966) which were used to express the recombinant protein. Transformed cells were selected on LB agar plate containing 100mg/ L ampicillin. Eight colonies were picked and screened by PCR using oligos HMOL 643 and HMOL 646 as primers (Fig. 21). Five out of the eight candidates gave positive results in the PCR screening.

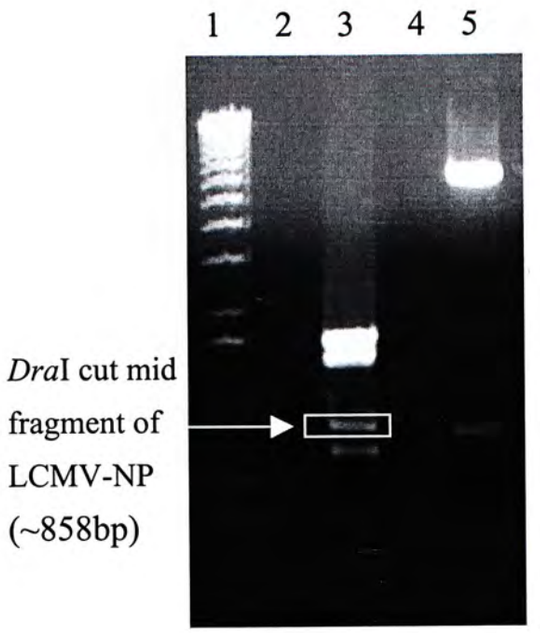


Fig. 19. *Dra*I digestion of pET-30a (+) - LCMV-NP and pBluescript II KS (+) - LCMV-NP. Lane 1: 1kb ladder; lanes 3: pBluescript II KS (+) - LCMV-NP; and lane 5: pET-30a (+) - LCMV-NP respectively; lanes 2 and 4: blanks.

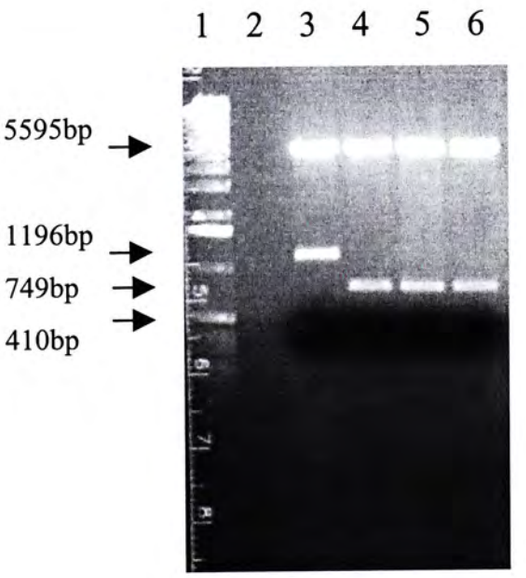


Fig. 20. *Bgl*II digestion of the four putative pET-30a (+) - LCMV-NP candidates. Lane 1: 1kb ladder; lane 2: blank. Lane 3-6: sample candidates. Lane 3: candidates gave bands of 1196bp, 410bp and 5595bp indicating successful subcloning; lanes 4-6: candidates gave bands of 749bp and 5595bp indicating no insert in pET30a(+).

Fig. 21. PCR screening of DE3/ pET-30a (+) - LCMV-NP. Positive candidates were screened by PCR method by using oligos HMOL 643 and HMOL 646 as primers. Lanes 1-8: samples under test. Lane 9: negative control with empty pET-30a(+); lanes 10 and 11: blanks; and lane 12: 1kb ladder (Gibco). Candidates in lanes 3,4,5,7 and 8 gave bands of expected size (1.7kb).

In order to synthesize the recombinant LCMV-NP in the bacterial system constructed, induction test was performed as described in the Materials and Methods Section (Fig. 22).

Detectable amounts of LCMV-NP protein were observed 1.5 hour after IPTG induction and leveled off at 3 hours after induction. Therefore, large-scale purification of the recombinant LCMV-NP protein was performed by harvesting cells grown for 3-4 hours after IPTG induction. His-tagged LCMV-NP recombinant fusion protein was then purified with prewashed Talon resin as described in the Materials and Methods Section. Fifteen microlitres of the four washing wastes and the two elutions were run on a 10% SDS-PAGE gel electrophoresis (Fig. 23). Purification was shown to be successful (Lanes 8 and 9 of Fig. 23).

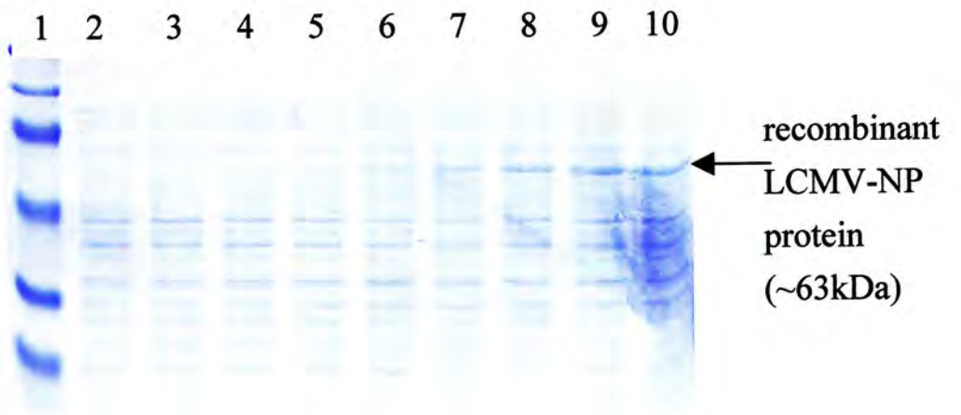


Fig. 22. Induction of LCMV-NP protein production in DE3 cells. Total proteins from bacterial cultures were loaded onto a 10% SDS-PAGE gel as described in the Materials and Methods Section. Lane 1: Pre-stained protein marker (Bio-Rad); lanes 2-10: samples harvested at time intervals: 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 hours after IPTG induction, respectively. A band of the expected size (about 63kDa) was observed from 1.5 hours and onwards after IPTG induction.

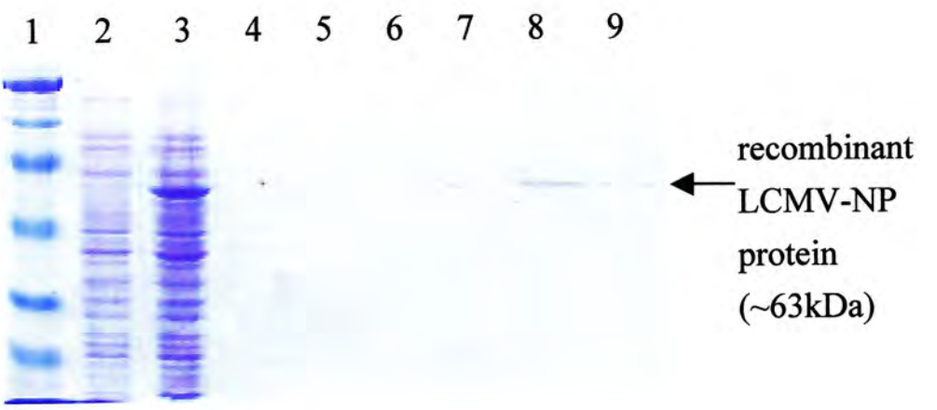


Fig. 23. Purification of the His-tagged LCMV-NP fusion protein. The SDS-PAGE gel showing the absence of recombinant LCMV-NP in washing wastes and the yield of first and second elutions. Lane 1: Pre-stained protein marker (Bio-Rad); lanes 2 and lane 3: raw culture samples before and after induction. Lanes 4-7: washing waste 1, 2, 3, 4 and lanes 8 and 9: the first and second elutions. Bacterial cultures were first harvested and lysed, and the total proteins were purified using prewashed Talon resin as described in the Materials and Methods Section.

3.1.1.6 Detection of recombinant LCMV-NP protein in transgenic *A. thaliana*

Total crude protein extract from LCMV-NP transgenic *Arabidopsis thaliana,* HML 603 A-1, HML 603 A-3, HML 603 C-2, HML 603 D-1-1, HML 603 D-1-2, HML 603 D-1-3, HML 603 D-1-4, HML 604 C-1-1 and HML 604 C-1-2, were supplied to Western blot and protein dot blot analyses. The concentrations of total crude protein extracts were estimated using Bio-Rad Bardford Protein Assay as described in the Materials and Methods Section. A standard curve was showed in Table 15 and Fig. 24. Twenty microlitres aliquots of total crude protein extracts from each transgenic line was added to 980 µl diluted dye. After recording the sample reading O.D.595, the protein concentrations of samples were estimated from the standard curve (Table 16).

Table 15 Raw data for the standard curve in Fig. 24.

BSA conc. (mg/ ml)	**O.D.595 Absorbance**
0.0	0.000
0.1	0.101
0.2	0.235
0.4	0.498
0.6	0.696
0.8	0.930
1.0	1.163

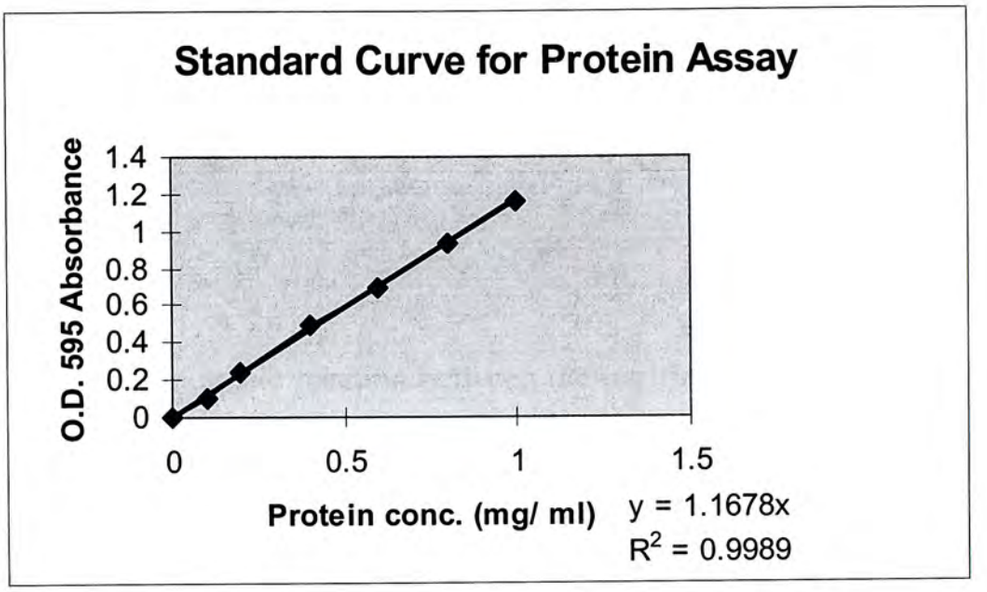


Fig. 24. Standard curve for protein assay. The standard curve for protein assay was generated by BSA standards of known concentrations by the Bardford method. Raw data was shown in Table 15.

Table 16 Concentration of crude protein extracts from LCMV-NP transgenic lines.

Samples	O.D. 595 Absorbance	Protein conc. (mg/ ml)
HML 603 A-1	0.698	0.598
HML 603 A-3	0.713	0.611
HML 603 C-2	0.797	0.682
HML 603 D-1-1	0.555	0.475
HML 603 D-1-2	0.604	0.517
HML 603 D-1-3	0.807	0.691
HML 603 D-1-4	0.527	0.451
HML 604 C-1-1	0.469	0.402
HML 604 C-1-2	0.671	0.575
Col-0	0.941	0.806
V7	0.362	0.310

For Western blot analysis, polyclonal antibodies against LCMV (from Dr. M. F. Saron of Pasteur Institute, France) were purified from the ascites fuild with MAb Trap GII (Pharmacia) as described in the Materials and Methods Section, and used as the primary antibody.

To test the immunogenic reaction between the purified primary antibody and purified His-tagged LCMV-NP from DE3 cells, dot blot analysis was performed. Serial dilutions of primary antibody were tested to estimate the minimum quantity that gave signal to the recombinant LCMV-NP produced in DE3 cells. The minimum titer was estimated to be 1:50 (Fig. 25).

To test if the LCMV-NP was produced in transgenic *A. thaliana*, transgenic lines including those containing single, double and multiple inserts were subjected to Western blot analysis. Approximately 7.5μg of total crude protein extract from each transgenic lines was run onto a 10% SDS-PAGE gel. Protein samples of *A. thaliana* transformed with the empty vector V7 (HML 539) and untransformed wild-type (Col-0) were used as the negative controls (Fig. 26). The result of western was shown Fig. 26. While a clear signal was obtained from the positive control (LCMV - NP produced in DE3 cells), all transgenic lines gave negative result, implying that the products of LCMV-NP in the lines were below the detection level of Western blot analysis.

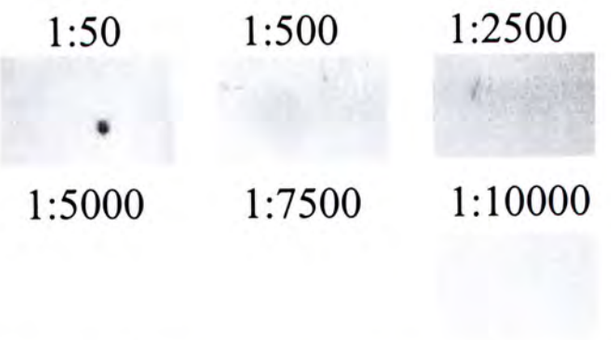


Fig. 25. Validity test of the purified anti-LCMV antibodies, the purified His-tagged LCMV-NP from DE3 cells (see the Materials and Methods Section) was dotted on the PVDF membrane (Bio-Rad). Serial dilutions of the purified anti-LCMV antibodies were added. The procedure of this protein dot blot analysis was described in the Materials and Methods Section.

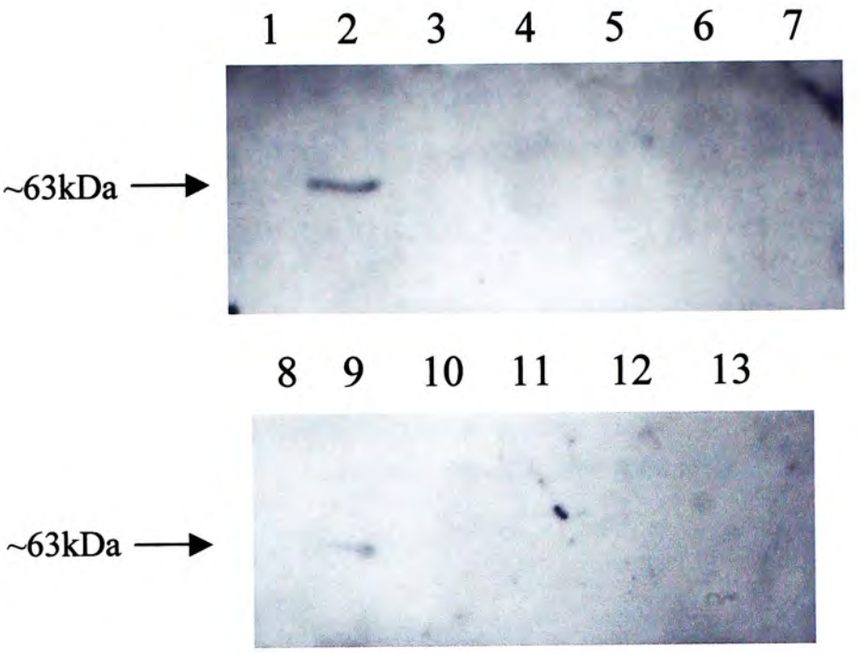
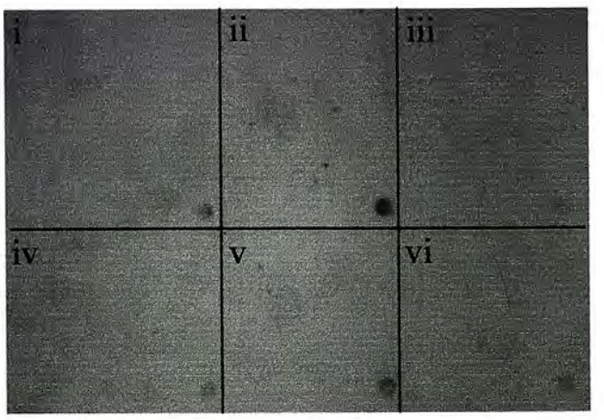


Fig. 26. Western blot analysis of recombinant LCMV-NP from transgenic *Arabidopsis thaliana*. Lanes 1: protein extracted from *A. thaliana* transformed with an empty V7 vector; lane 8: untransformed wild-type Col-0; lanes 2 and 9: recombinant LCMV-NP produced in DE3 cells; lanes 3, 4, 5, 6, 7, 10, 11, 12 and 13: proteins extracts from HML603A-1, HML603A-3, HML603C-2, HML603D-1-1, HML603D-1-2, HML603D-1-3, HML603D-1-4, HML604C-1-1 and HML604C-1-2 respectively. The detailed procedure for Western blot analysis was described in the Materials and Methods Section.

To maximize the amount of protein samples that could be loaded for detection, protein dot blot was adopted. A series of protein extract of different amounts (9, 18, 27, 36, 45, 54 μg) were applied to a PVDF membrane (Fig. 27). However, no positive signals were obtained from any of the dot blot analyses. The positive control (recombinant LCMV-NP from DE3 cells), on the other hand, gave clear signal. When 18μg or more of the crude proteins were added to the PVDF membrane, a white color was observed on the exposed film, indicating that the membrane was overloaded with sample.

(a)



(b)

①	②	③		
④	⑤	⑥	⑦	
⑧	⑨			
⑩	⑪		⑫	

Fig. 27. Protein dot blot analysis of recombinant LCMV-NP from transgenic *Arabidopsis thaliana*. (a) Panels i, ii, iii, iv, v and vi contained of 9, 18, 27, 36, 45 and 54 μg of proteins, respectively. The sample co-ordinates were the same for each panel and were summarized in (b). Spots 1-9: proteins extracts from HML603 A-1, HML603 A-3, HML603 C-2, HML603 D-1-1, HML603 D-1-2, HML603 D-1-3, HML603 D-1-4, HML604 C-1-1, HML604 C-1-2, HML359 (*A. thaliana* transformed with an empty V7 vector) and Col-0 (untransformed wild-type), respectively; spot 12: positive control (recombinant LCMV-NP protein from DE3 cells).

3.1.2 Expression of goldfish growth hormones I and II (GHI and GHII) in transgenic *Arabidopsis thaliana*

3.1.2.1 Construction of transgenic *Arabidopsis thaliana* expressing genes encoding the goldfish (*Carassius auratus*) growth hormones I and II

Goldfish (*Carassius auratus*) growth hormones I and II (GHI and GHII) full length cDNA clones (CAGHa I and CAGHb I) were obtained from Dr. K. L. Yu (HKU) via Dr. W. Ge (CUHK). The original clones were in vector pBluescript II KS (-) and the restriction maps were shown in Fig. 28 and Fig. 29. Primary transformants of GHI and GHII transgenic plants were constructed previously by Dr. Hon-Ming Lam and Mr. Jack Lo (CUHK). In this experiments, GHI and GHII cDNAs were first subcloned into the *Xho*I and *Xba*I sites of V7 vector (Fig. 30 and Fig. 31) and then electroporated into the *Agrobacterium* GV3101/ pMP90.

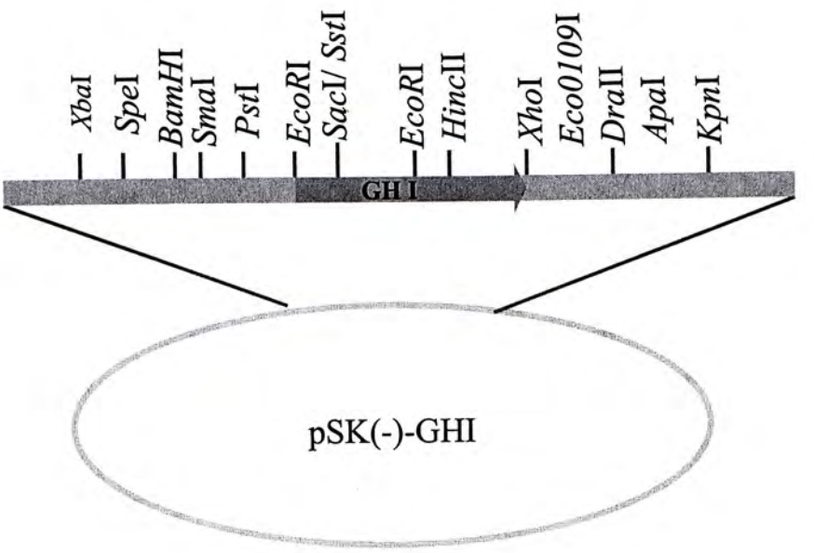


Fig. 28. The schematic diagram of pBluescript II SK (-) - GHI.

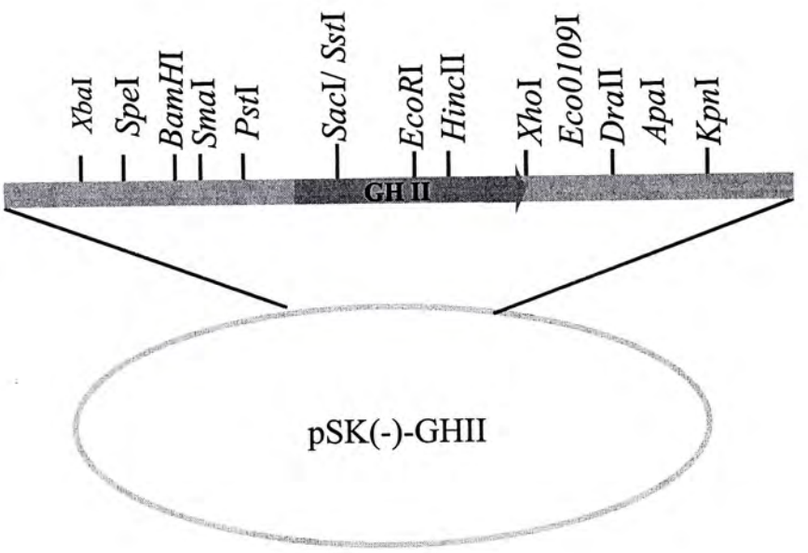


Fig. 29. The schematic diagram of pBluescript II SK (-) - GHII.

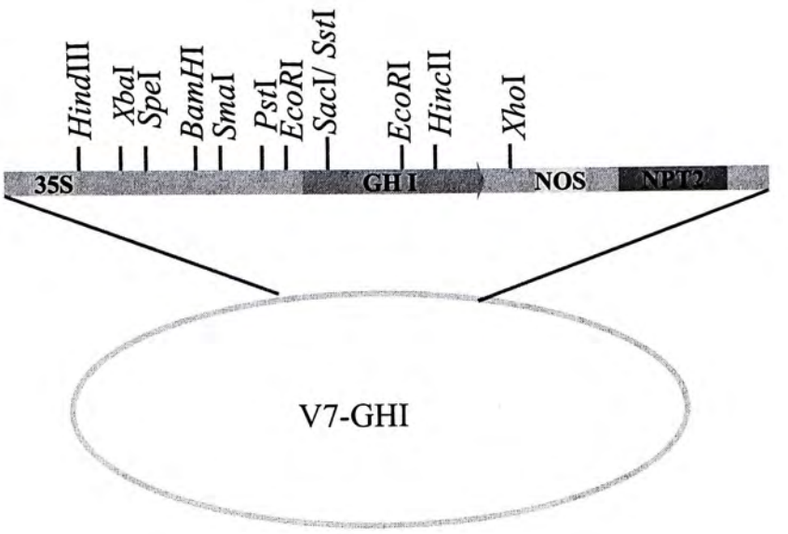


Fig. 30. The schematic diagram of V7-GHI.

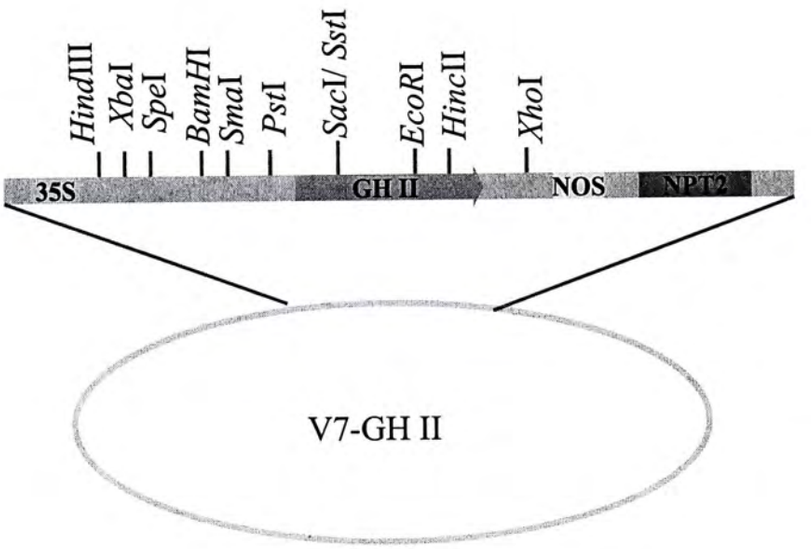


Fig. 31. The schematic diagram of V7-GHII.

Transgenic *Arabidopsis thaliana* was constructed by *Agrobacterium*-mediated transformation using vacuum infiltration technique (see the Materials and Methods Section). A total of twelve plants were used for each of the GHI or GHII constructs. Four (HML555-1, HML555-2, HML555-3 and HML555-4) and three (HML554-1, HML554-3 and HML554-7) independent transformants were obtained for GHI and GHII, respectively.

Heterozygous lines were used to determine the number of insertion loci by Chi-square analysis. Transgenic lines each containing a single insertion locus were selected and allowed to grow and shed seeds. About 100 T_3 seeds from each lines were further screened on MS agar plate plus 50mg/ L kanamycin. Homozygous lines were identified in case all T_3 progenies were kanamycin resistant. They were HML 554 1-3, HML 554 3-1, HML 554 3-3, HML 554 3-4, HML 554 4-1 and HML 554 4-5 for GHII transgenic plants. Among the six transformants, HML 554 3-1, HML 554 3-3 and HML 554 3-4 were from the same parent and HML554 4-1 and HML 554 4-5 from another. GHI transgenic plants included HML 555 1-3, HML 555 1-6, HML 555 3-4 and HML 555 7-4, while HML 555 1-3 and 1-6 come from the same primary transgenic line (Table 17).

Table 17 Chi-square test for a 3:1 green: yellow ratio of selfed progenies of primary GHI and GHII transformants[(a)]

a) HML 554 (GHII)

LINES	# Green	# Yellow	X^2 value	Conclusion
HML 554-1	88	20	2.42	single insertion locus
HML 554-2	almost all were yellow, few were green. This one was rejected.			
HML 554-3	132	38	0.63	single insertion locus
HML 554-4	91	20	3.72	single insertion locus

b) HML 555 (GHI)

LINES	# Green	# Yellow	X^2 value	Conclusion
HML 555-1	99	36	0.17	single insertion locus
HML 555-3	111	28	1.75	single insertion locus
HML 555-7	127	43	0.01	single insertion locus

[a]Significance level was set of 0.05 and the critical value was calculated as 3.841.

3.1.2.2 Southern blot and Northern blot analyses of transgenic plant containing the GHI and GHII cDNA

T_3 plants from the six and four homozygous transformants of GHI and GHII, respectively, were grown for 4 weeks. DNA and RNA samples were extracted as described in the Materials and Methods Section.

To perform Southern blot and Northern blot analyses, DIG-labelled cRNA

probes of transgenes were prepared. Since the nucleotide sequence homologies of goldfish GHI and GHII were very high (93% identity), GHI cRNA probe was used to hybirdize both GHI and GHII transgenes. To generate cRNA probes for GHI, 2μg pBluescript II SK (-) -GHI was digested with *Xba*I to linearize the plasmid at the 5' end of the insert prior to *in vitro* transcription. The linearized fragment was purified with Bio-Rad Prep-A-Gene Purification kit. *In vitro* transcription was accomplished with DIG RNA labeling kit (Roche). In the *in vitro* transcription reaction, 1μg linearized and purified pBluescript II SK (-) - GHI was added in the reaction mixes as described in the Materials and Methods Section.

In Southern blot analysis, approximately 1g plant tissue from each of the homozygous GHI and GHII transgenic *Arabidopsis thaliana* with a single insertion locus was used to extract total genomic DNA. Three independent transformants from each of the GHI and GHII transgenic plants were employed (HML 554 1-3, HML 554 3-1, HML 554 3-3, HML 554 3-4, HML 554 4-1, HML 554 4-5, HML 555 1-3, HML 555 1-6, HML 555 3-4 and HML 555 7-4) (see the Materials and Methods Section). Twenty microgram of genomic DNA from each sample was digested with *Xba*I (37°C), *Sma*I (25°C) and *EcoR*V (37°C) overnight. Southern blot analysis was performed as described in the Materials and Methods Section.

Complete digestion of genomic DNA sample were confirmed by ethidium bromide staining (Fig. 32(a)). Southern blot analysis showed positive signals for all selected lines. However, HML 554 3-4 and 4-1 gave an extra band that might be due to incomplete digestion of samples.

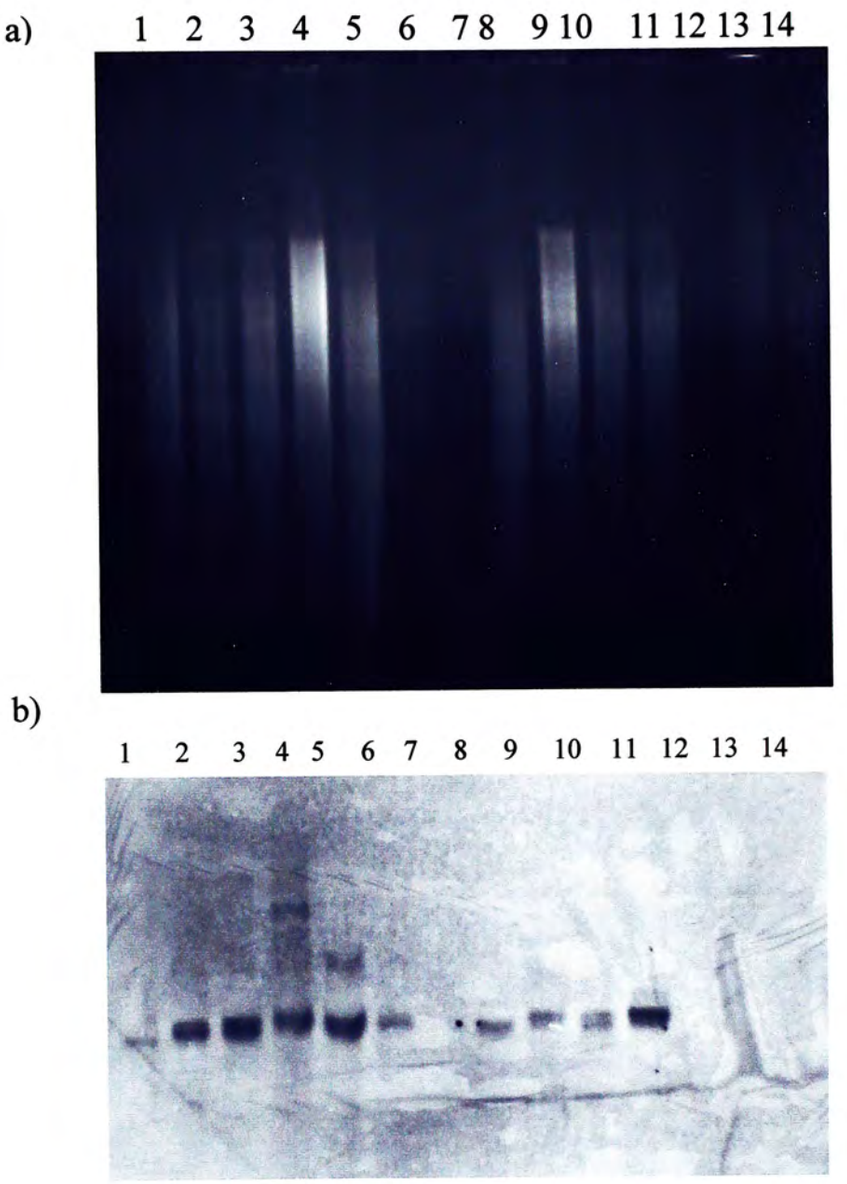


Fig. 32. Southern blot analysis of GHI and GHII transgenic *Arabidopsis thaliana.* (a) Ethidium bromide staining of genomic DNA digested with *Xba*I, *Sma*I and *EcoR*V. (b) Autoradiograph of Southern blot. Lanes 1-6: HML 554 1-3, HML 554 3-1, HML 554 3-3, HML 554 3-4, HML 554 4-1 and HML 554 4-5; lanes 8-11: HML 555 1-3, HML 555 1-6, HML 555 3-4 and HML 555 7-4; lanes 7 and 12: blanks; lanes 13 and 14: negative controls including transformant containing T-DNA from the empty V7 vector (HML359) and untransformed wild-type (Col-0), respectively.

Total RNA of homozygous GHI and GHII transgenic plants were also extracted and used for Northern blot analysis. Approximate 5 g plant tissue of homozygous goldfish GHI and GHII transgenic *A. thaliana* was used to extract total RNA. The cRNA probes based on GHI was used to detect messenges from both GHI and GHII (93% DNA identity) in transgenic lines (Fig. 33). Northern blot analysis showed that high level of GHI transgene expression occurred in all transgenic lines selected. On the other hand, only the HML 554 1-3 line gave a strong signal for GHII transgenic line. Other GHII transgenic lines exhibits low level of transgene expression. Similar result was obtained with cRNA probes based on GHII were used instead (Fig. 34).

3.1.2.3 Detection of recombinant GHI and GHII from transgenic *A. thaliana*

Total crude proteins from homozygous GHI and GHII transgenic *A. thaliana* were extracted with 1X PBS (pH 7, the physiological pH for growth hormones). The quantity of proteins extracted estimated by Bio-Rad Bardford protein assay. A standard curve was constructed to estimate the total protein concentration in plant extracts (Table 18 and Fig. 35). Twenty microlitres total crude protein extracts extracted from each transgenic lines was added to 980 µl diluted dye. The protein concentrations of samples were estimated using the standard curve (Table 19).

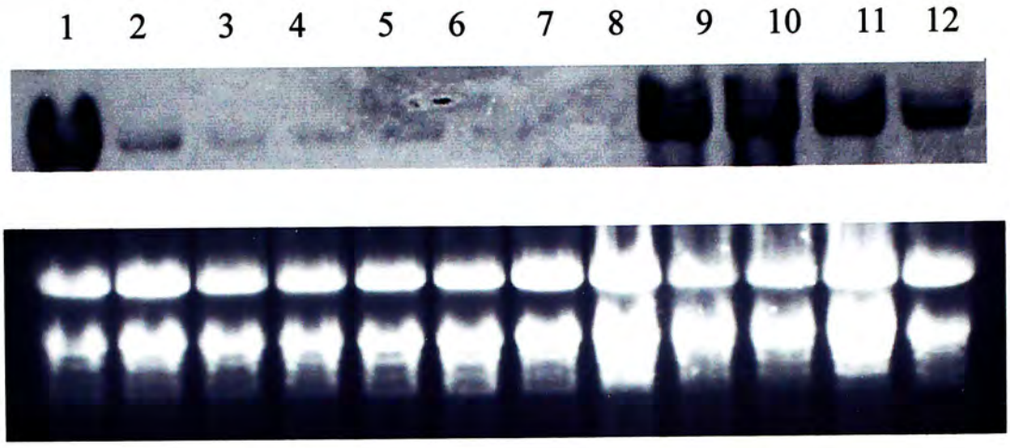


Fig. 33. Northern blot analysis of homozygous GHI and GHII transgenic *A. thaliana* with GHI cRNA probes. Total RNA of each transgenic lines was extracted and the cRNA probe was generated based on GHI gene as described in the Materials and Methods Section. Lanes 1-6: RNA samples from GHII transgenic plants (HML 554 1-3, HML 554 3-1, HML 554 3-3, HML 554 3-4, HML 554 4-1 and HML 554 4-5); lane 7: untransformed wild-type (Col-0); and lanes 8-12: RNA samples from GHI transgenic plants (HML 555 1-3, HML 555 1-6, HML 555 3-4 and HML 555 7-4).

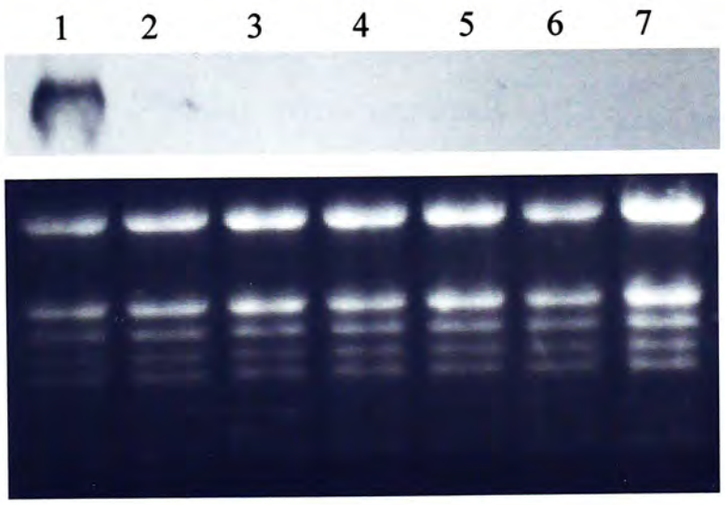


Fig. 34. Northern blot analysis of homozygous GHII transgenic *A. thaliana* using GHII cRNA probes (see the Materials and Methods Section). Lanes 1-6: RNA samples from GHII transgenic plants (HML 554 1-3, HML 554 3-1, HML 554 3-3, HML 554 3-4, HML 554 4-1 and HML 554 4-5); and lane 7: untransformed wild-type (Col-0).

Table 18 Raw data for the standard curve in Fig. 35.

Protein conc. (1 mg/ ml)	Absorbance O.D. 595
0.0	0.000
0.1	0.053
0.2	0.070
0.4	0.173
0.6	0.253
0.8	0.350

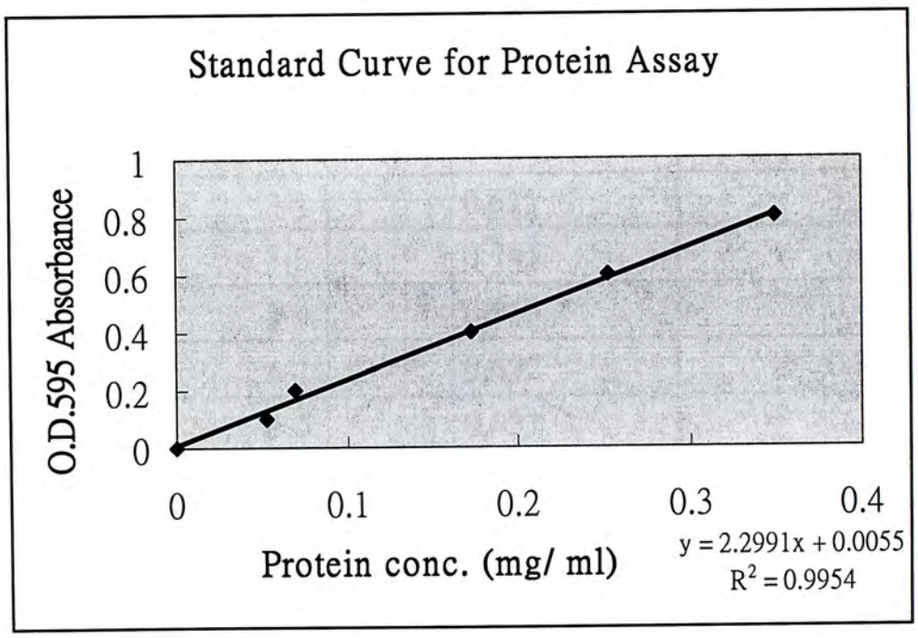


Fig. 35. Standard curve for protein assay. The standard curve for protein assay was generated by BSA standards of known concentrations by the Bardford method. Raw data was shown in Table 18.

Table 19 Concentration of crude protein extracts from GHI and GHII transgenic lines.

Samples		Absorbance O.D. 595	Protein conc. (mg/ml)
High salt 554	1-3	0.369	0.158
554	3-1	0.251	0.108
554	3-3	0.465	0.200
554	3-4	0.315	0.135
554	4-1	0.409	0.176
554	4-5	0.521	0.224
555	1-3	0.682	0.294
555	1-6	0.598	0.258
555	3-4	0.286	0.122
555	7-4	0.483	0.208
	Col-0	0.387	0.166
Low salt 554	1-3	0.551	0.237
554	3-1	0.611	0.263
554	3-3	0.741	0.320
554	3-4	0.933	0.403
554	4-1	0.692	0.299
554	4-5	0.837	0.362
555	1-3	0.593	0.256
555	1-6	0.272	0.116
555	3-4	0.682	0.294
555	7-4	0.272	0.116
	Col-0	0.853	0.369

Total crude proteins from the transgenic lines were extracted by first using low salt buffer, followed by re-extracted of the residues with high salt buffer (see the Materials and Methods Section). About 5μg of high salt or low salt crude protein extracts from each transgenic line was used in the radioimmunoassy (assayed by Dr. A. O. L. Wong of The University of Hong Kong). In Fig. 36, the data of Northern blot analysis and radioimmunoassay were presented in parallel. The results did not show a positive correlate between the RNA levels and protein quantities.

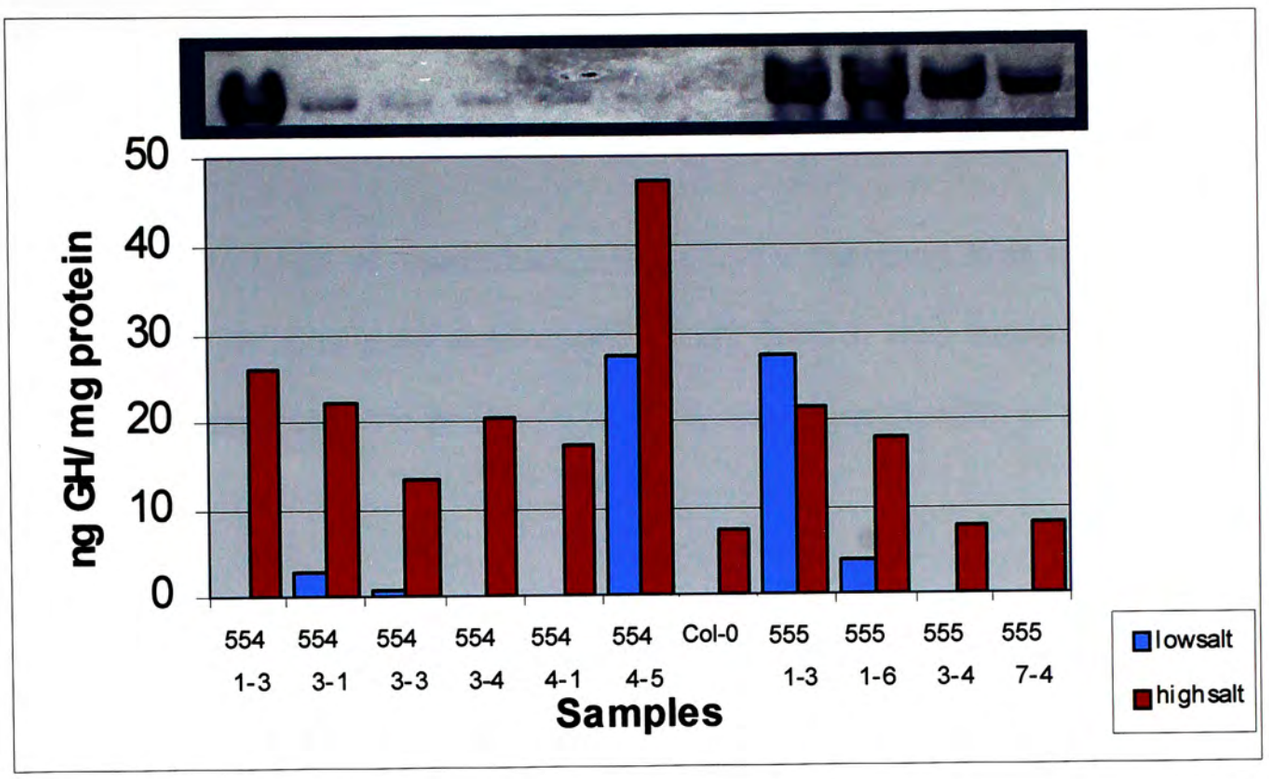


Fig. 36. Immunoassay of recombinant GHI and GHII from transgenic *A.thaliana*. The bars in red representing the signal of growth hormones detected in high salt extracted samples, whereas bars in purple showed the level of growth hormones detected in low salt extracted samples. The panel above the bar graph showed the corresponding Northern blot result of GHI and GHII transgenic *A. thaliana* detected with the GHI cRNA probes.

3.2 *In vitro* transcription and translation of target genes in rabbit reticulocyte and wheat germ systems

Since the levels of recombinant proteins in the transgenic lines were either low (GHI and GHII) or even undetectable (LCMV-NP), *in vitro* transcription and translation experiments were performed to verify translation capacity of the cDNA constructs.

The pGEM-3Zf(+) vector was employed for this purpose.

3.2.1 Subcloning the target genes into pGEM-3Zf(+) vector

3.2.1.1 Subcloning of the LCMV-NP cDNA fragment into pGEM-3Zf(+) vector

The LCMV-NP cDNA fragment was cut out from pBluescript II KS (+) - LCMV-NP using *Xho*I and *Xba*I, while 1 μl pGEM-3Zf(+) was restricted with *Xba*I and *Sal*I. Since *Sal*I and *Xho*I produced the same compatible end, the ligation between teh digested LCMV-NP cDNA fragment and the restricted pGEM-3Zf(+) resulted in a loss of both the *Xho*I and *Sal*I. The procedure of ligation and transformation were described in the Materials and Methods Section.

Eight colonies were picked and screened with CloneChecker™ system (GibcoBRL) based on the company's instruction (Fig. 37). All eight colonies showed positive results. Their sequence and orientation were further confirmed by partial sequencing with the T7 primer. The schematic representation was shown in Fig. 38.

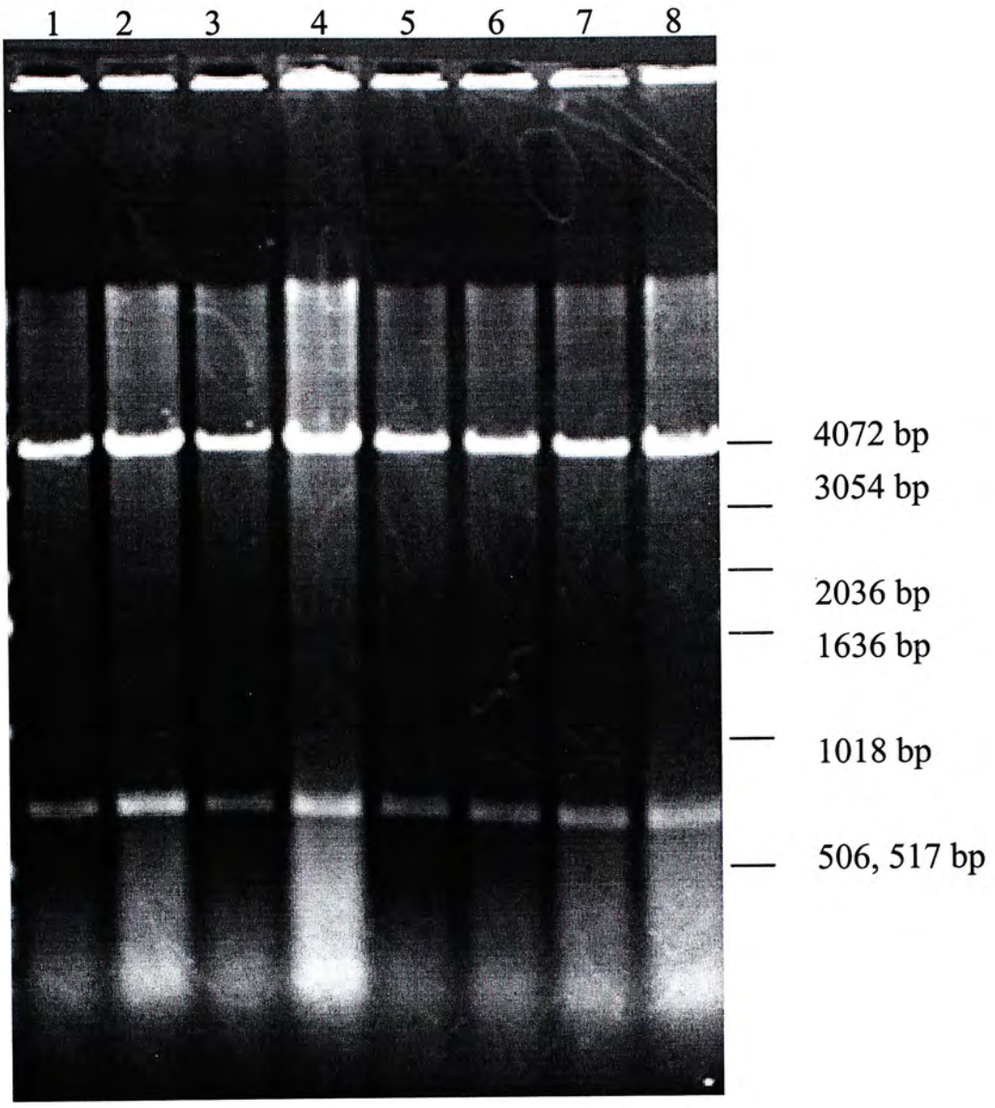


Fig. 37. Screening of pGEM-3Zf (+)-LCMV-NP candidates. Candidates clones were digested with *Pst*I and screening with Clonechecker system. All eight samples gave positive results with a ~ 4kb band and a ~ 700bp band.

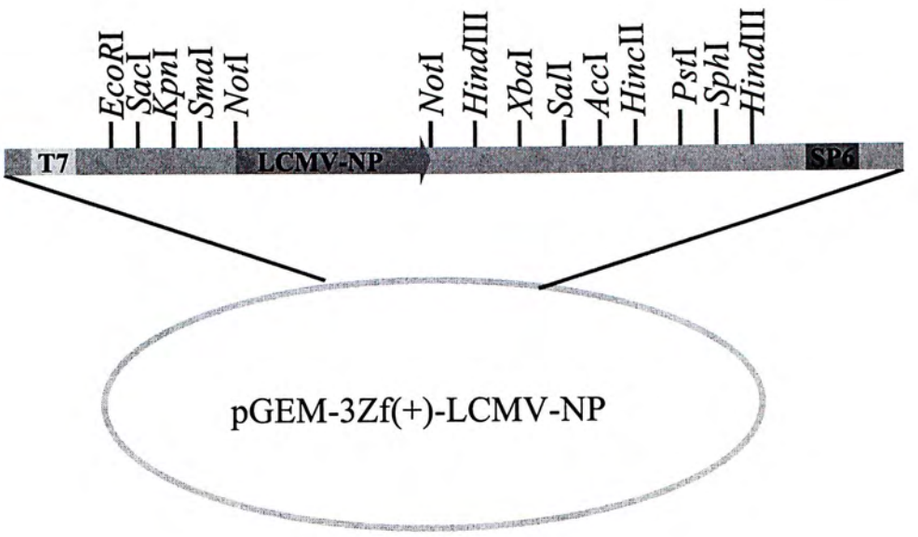


Fig. 38. The schematic diagram of pGEM-3Zf (+)-LCMV-NP.

3.2.1.2 Subcloning of goldfish GHI and GHII fragments into pGEM-3Zf(+) vector

Goldfish GHI and GHII cDNA fragments were cut out from 1μg pBluescript II KS(-)-GHI and pBluescript II KS(-)-GHII plasmids using *Xho*I and *Xba*I. About 1 μg pGEM-3Zf(+) vector was also digested by *Xho*I and *Xba*I enzymes at 37°C overnight. The target gene fragments and digested vector were gel purified using the Bio-Rad Prep-A-Gene DNA Purification kit. The ligation mixture of the target gene fragments and the digested vector was transformed into DH5α as described in the Materials and Methods Section. The transformed cells were spread on LB agar plates containing with 100mg/ L ampicillin for selection.

Two colonies of DH5α/ pGEM-3Zf(+) -GHI and four colonies of DH5α/ pGEM-3Zf(+) -GHII were picked and inoculated into 5 ml LB broth containing 100 mg/L ampicillin. The plasmids purified with Wizard Plus Minipreps DNA Purification system (Promega). Plasmid DNA obtained from each candidate was digested with *BamH*I and *Hind*III, (Fig. 39). The four candidates of DH5α/ pGEM-3Zf(+) -GHII were all positive clones, whereas one of the two candidates of DH5α/ pGEM-3Zf(+) -GHI showed positive result.

For the pGEM-3Zf(+) -GHI construct, no suitable restriction site was available to linearize the plasmid at the 3' end of the GHI cDNA . Therefore, the GHI cDNA fragment was cut out with *Pst*I and religated into the vector and screened for new insertions at opposite orientation. Twelve colonies were selected and inoculated into 5ml LB broth containing 100mg/ L ampicillin. After purification of plasmid using the Minipreps DNA Purification system (Promega), the DNA plasmid from each candidate was digested with *Hind*III (Fig. 40). Three out of the twelve

candidates contained inserts in the desired orientation (*Hind*III cut gave a 4kb and a 300bp band). The schematic diagrams of the final construction of pGEM-3Zf(+)-GHI and pGEM-3Zf(+)-GHII were shown in Fig. 41 and Fig. 42.

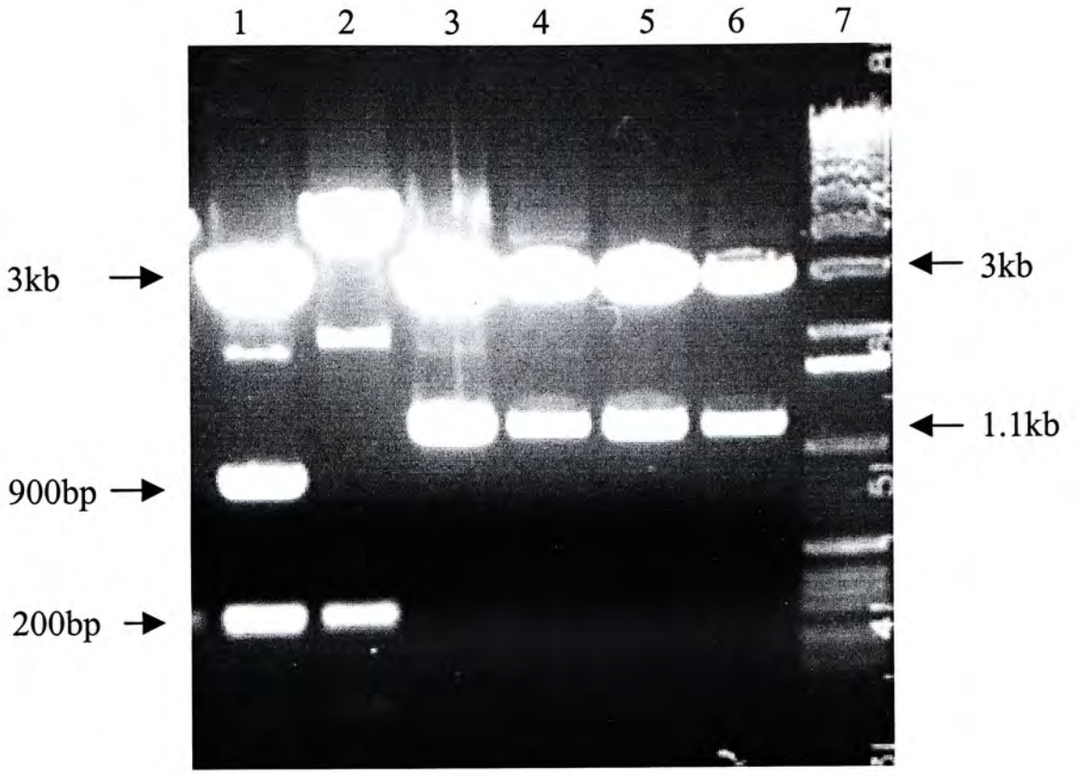


Fig. 39. Screening of pGEM-3Zf (+)-GHI and pGEM-3Zf (+)-GHII candidates. Plasmids prepared from the candidate clones were digested with *BamH*I and *Hind*III. Positive clones for pGEM-3Zf (+)-GHI should give bands of 3kb, 900bp and 200bp, while that of pGEM-3Zf (+)-GHII should give bands of 3kb and 1.1kb. Lanes 1 and 2: pGEM-3Zf (+)-GHI candidates; lanes 3-6: pGEM-3Zf (+)-GHII; lanes 7: 1kb DNA ladder (GibcoRBL). The 1.6kb extra band in lane 1 was probably due to incomplete digestion.

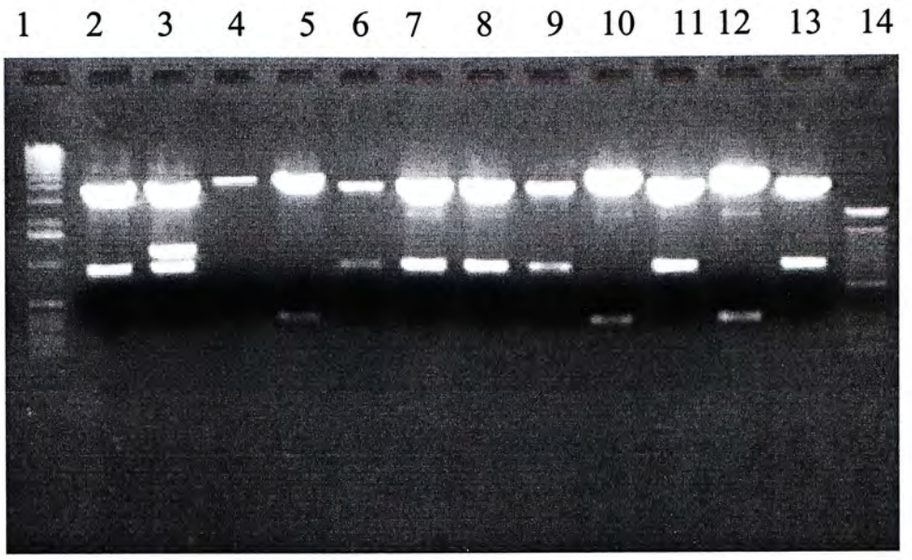


Fig. 40. Changing orientation of GHI cDNA insert in the pGEM-3Zf (+)-GHI clone. Plasmids prepared from the candidate clones were digested with *Hind*III. Positive clones for pGEM-3Zf (+)-GHI with the GHI cDNA inserted in the desired orientation should give a 4kb and a 300bp band. Lanes 2-13: the candidates under test; lanes 1 and 14: 1kb DNA ladder and 100bp DNA ladder respectively (GibcoBRL). Lanes 5, 10 and 12 were positive subclones with the GHI cDNA insert in right orientation, showing bands of size about 4 kb and 300bp. Lanes 2, 3, 6-9, 11 and 13 were clones with the insert in wrong orientation. Lane 4 was a candidate without insert.

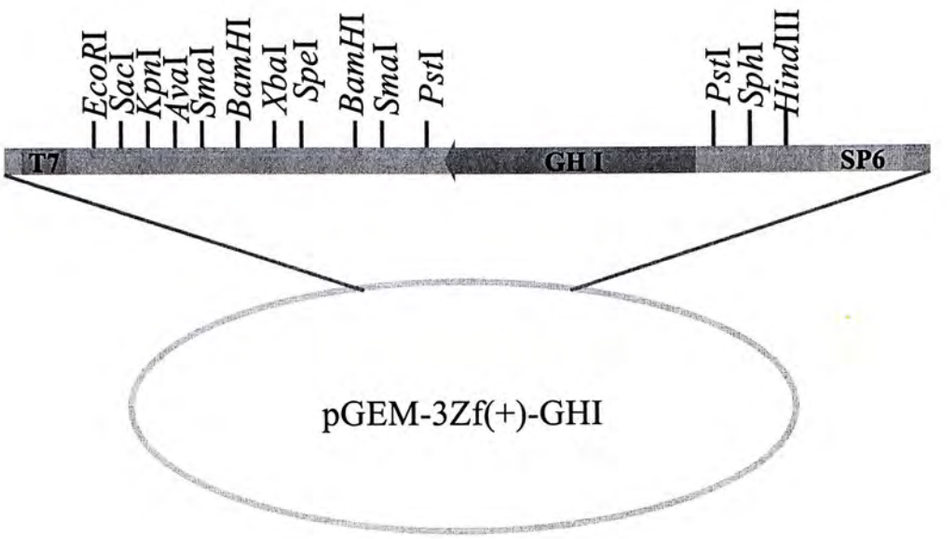


Fig. 41 The schematic diagram of pGEM-3Zf(+)-GHI.

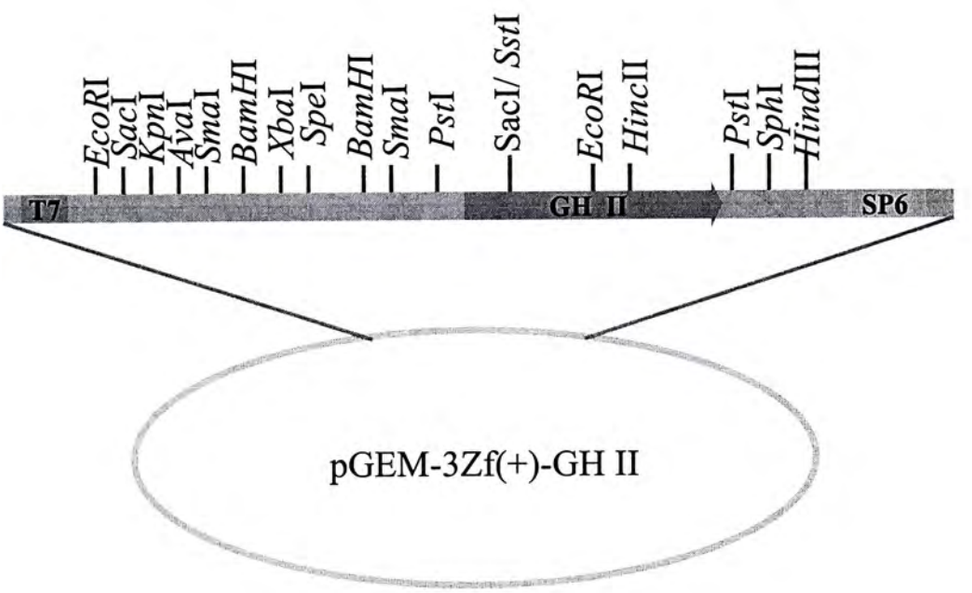


Fig. 42 The schematic diagram of pGEM-3Zf(+)-GHII.

3.2.2 *In vitro* transcription of target genes

To generate RNA transcripts of GHI, GHII and LCMV-NP *in vitro*, pGEM-3Zf(+) - GHI and pGEM-3Zf(+) - LCMV-NP constructs were digested with *Xba*I, while pGEM-3Zf(+) - GHII was restricted by *Hind*III at 37°C overnight. The digested samples were purified PCI extraction and subsequent ethanol precipitation as described in the Materials and Methods Section. The quantities of pGEM-3Zf(+) - GHI and pGEM-3Zf(+) -GHII were determined by spectrophotometry and gel electrophoresis (Fig. 43).

In vitro transcription of the GHI, GHII and LCMV-NP cDNA were performed using the Ribomix Large Scale RNA Production Systems-T7 and SP6 (Promega). SP6 RNA polymerase (for pGEM-3Zf(+)-GHI and pGEM-3Zf(+)-LCMV-NP) and T7 RNA polymerase (for pGEM-3Zf(+)-GHII) were used as described in the Materials and Methods Section (Fig. 44).

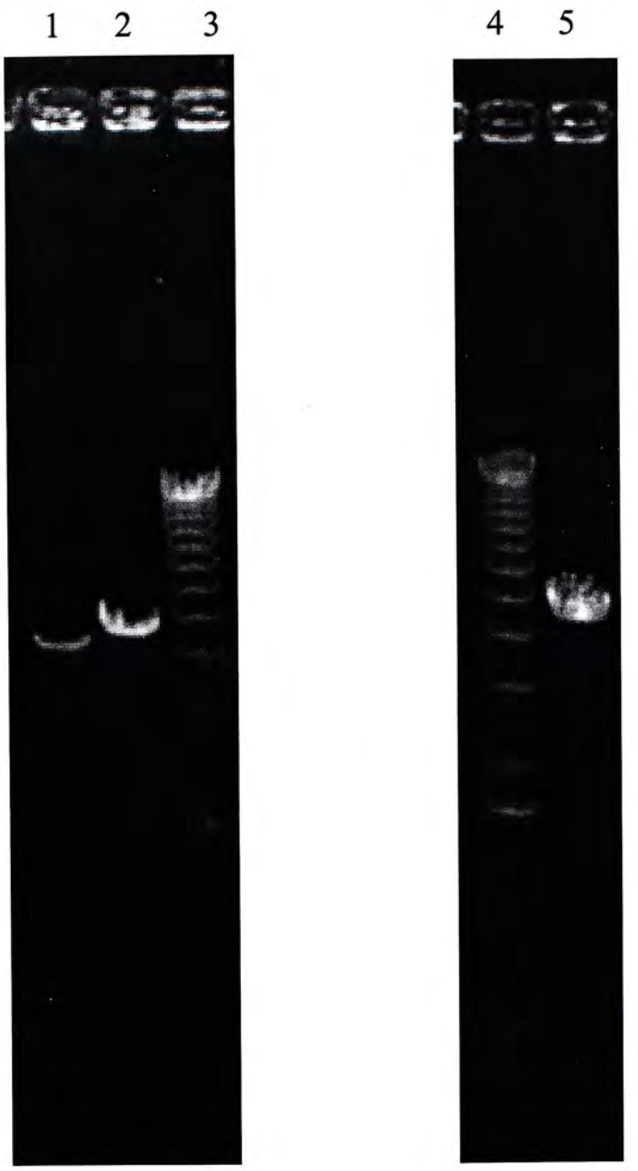


Fig. 43. Ethidium bromide gel showing the qualities and quantities of linearized pGEM-3Zf (+)-GHI, pGEM-3Zf (+)-GHII and pGEM-3Zf (+)-LCMV-NP. The recombinant plasmids were restricted as follows: pGEM-3Zf (+)-GHI and pGEM-3Zf (+)-LCMV-NP by *Xba*I; pGEM-3Zf (+)-GHII by *Hind*III. Lanes 1, 2 and 5: 1µl sample of linearized pGEM-3Zf (+)-GHI, pGEM-3Zf (+)-GHII and pGEM-3Zf (+)-LCMV-NP, respectively; lanes 3 and 4: 1kb DNA ladder (GibcoBRL).

Fig. 44. *In vitro* transcription of goldfish GHI and GHII and LCMV-NP cDNA. *In vitro* transcription was performed with the linearized plasmid clone shown in Fig. 43 using the Ribomix Large Scale RNA Production Systems - T7 and SP6 (Promega). Lane 1: GHI transcripts generated with SP6 RNA polymerase; lane 2: GHII transcripts generated with T7 RNA polymerase; lane 3: LCMV-NP transcripts generated with SP6 RNA polymerase. One microlitre of each reaction product was loaded onto a 1% denaturing RNA gel.

3.2.3 *In vitro* translation with rabbit reticulocyte lysate and wheat germ extract systems

LCMV-NP, GHI and GHII proteins were generated *in vitro* by adding the mRNA transcripts produced above to rabbit reticulocyte lysate or wheat germ extract systems. Transcend non-radioactive translation detection system (Chemiluminescent) (Promega) was used as described in the Materials and Methods Section. The translated products were then resolved in SDS-PAGE gel (10% SDS-PAGE gel for GHI and GHII proteins, and 15% SDS-PAGE gel for LCMV-NP protein) and transferred to PVDF membrane for detection. The results of *in vitro* translation with rabbit reticulocyte lysate and wheat germ extract were shown in Fig. 45.

Using *in vitro* transcription and translation, GHI and LCMV-NP genes were found to be strongly expressed, while GHII exhibited difficulty in expression in both rabbit reticulocyte lysate or wheat germ extract systems. For *in vitro* transcription of GHI in both rabbit reticulocyte lysate or wheat germ extract systems, a distinct and strong 23kDa band was observed. The signal for GHII was very weak (if any) in both systems. For *in vitro* translation of LCMV-NP, and distinct but weak 63kDa band was also observed in the rabbit reticulocyte lysate. In wheat germ extract system, a 63kDa band was also observed in the positive control, however, the intensity of the 63kDa band generated by the LCMV-NP mRNA was much stronger.

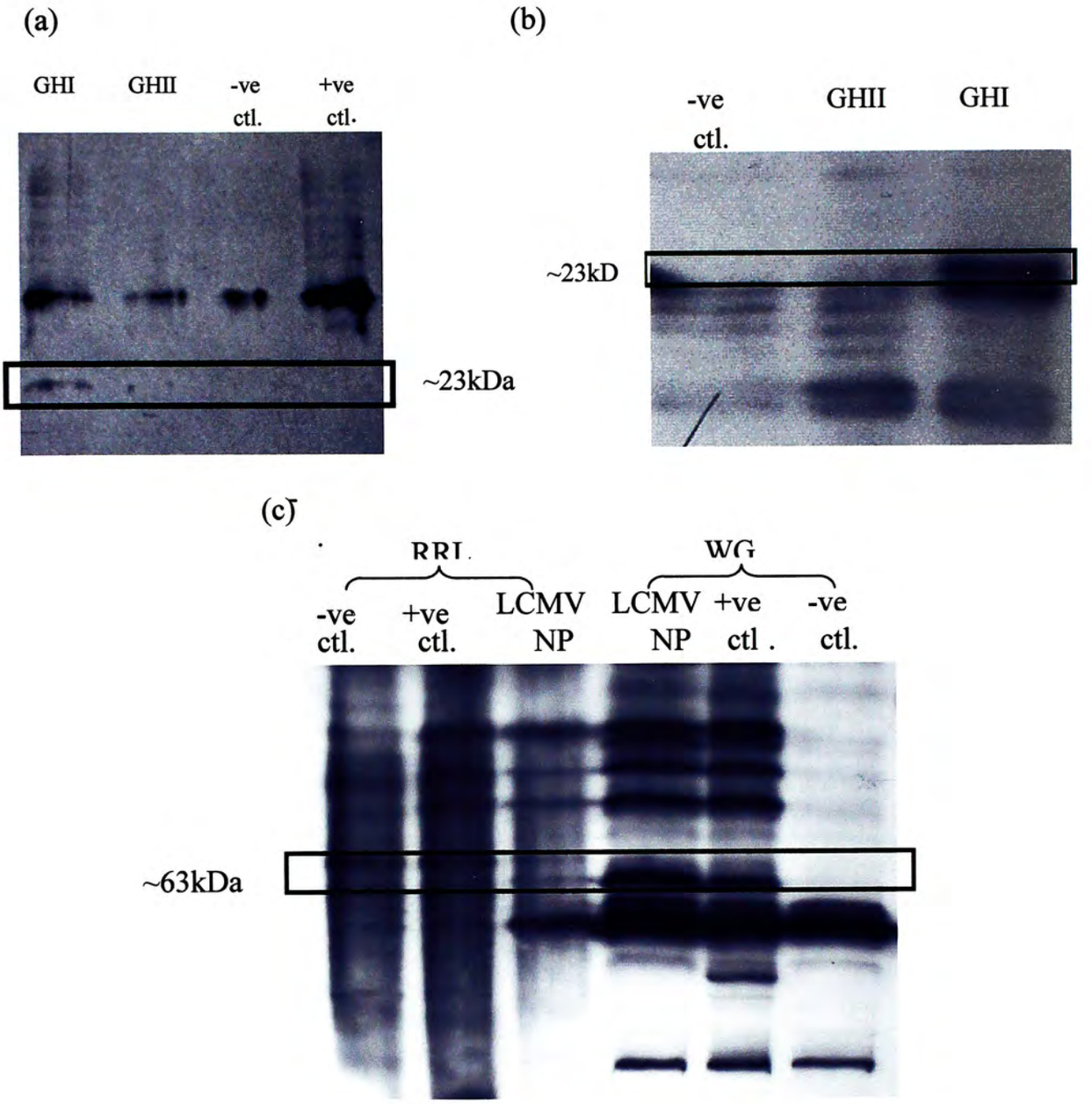


Fig. 45. *In vitro* translation of GHI, GHII and LCMV-NP mRNA. (a & b) *In vitro* translation of GHI and GHII using rabbit reticulocyte lysate and wheat germ systems, respectively. (c) *In vitro* translation of LCMV-NP using rabbit reticulocyte lysate and wheat germ systems. +ve ctl.: Positive controls of *in vitro* translation kits; -ve ctl.: negative control without mRNA addition.

3.3 Establishment of *Glycine max* regeneration and transformation systems

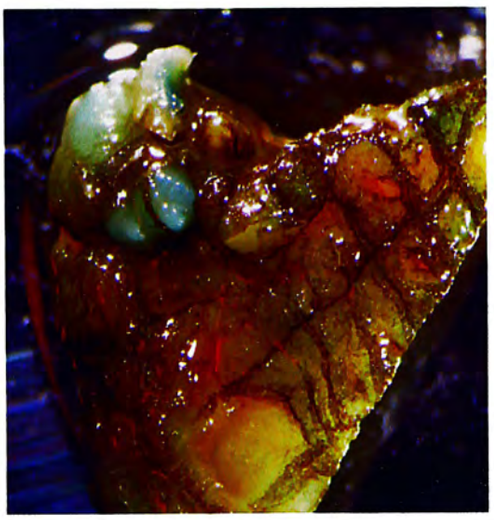
3.3.1 Establishment of regeneration system for soybean cotyledonary node explant

To establish the *Agrobacterium*-mediated soybean transformation platform of cotyledonary node explant (Trick *et al.*, 1997), soybean cotyledonary node explant tissue culture and regeneration system based on shoot morphogenesis had to be set up.

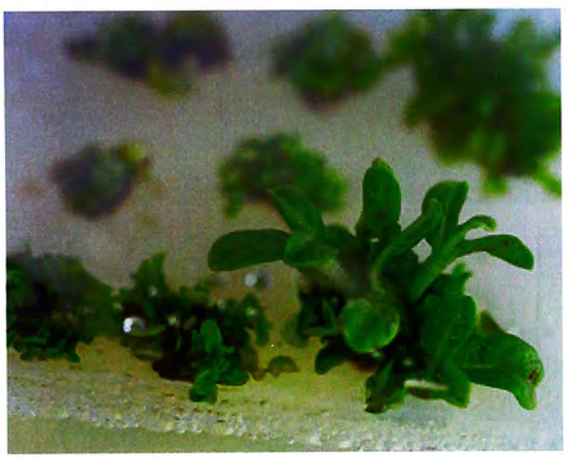
Six soybean varieties including Ji lin xiao li no. 1, He feng no. 25, Ji lin no. 30, Ji lin no. 36, Chang nong no. 5, Zhe chun no. 3 and Ai jiao zao were used as raw materials for regeneration and transformation. After surface sterilization, soybean seeds were allowed to germinate on MS medium. The seedlings were then used to prepare the cotyledonary node explants as described in the Materials and Methods Section. Typical result showing different stages of regeneration was presented in Fig. 46.

Following shoot induction was the root induction of the plantlets. Plantlets with shoot developed were placed in root stimulating medium and cultivated under the same condition. Root started to develop and shoot continued to differentiate in these plantlets (Fig. 47).

(a)



(b)



(c)

Fig. 46. Shoot induction of the cotyledonary node explants in differentiation medium. (a) Bud regenerated at the nodal region of the cotyledonary node explant and stained in blue by GUS activity. (b and c) Regenerated cotyledonary node explants with multiple shoots.

Fig. 47. Developing plantlets in root stimulating medium.

3.3.2 Establishment of soybean transformation system

Basic protocol of *Agrobacterium*-mediated transformation of soybean cotyledonary node explants was described in the Materials and Methods Section. To optimize the transformation system, effects of several factors, including plant hosts adopted, *Agrobacterium* strains used, application of vacuum infiltration, application of antibiotic selection, conditions of co-cultivation and application of detergent were tested.

3.3.2.1 Definition of transformation efficiency

Only quick assays were used to estimate the transformation efficiency. Initially, explants that could regenerate and differentiate multiple shoots in selection medium (containing 50mg/ L kanamycin) were considered as positive transformants (Fig. 48). However, subsequent studies suggested that inclusion of kanamycin severely imhibited the regeneration process (see below). Therefore, assay for GUS activity by 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (X-gluc) staining was employed in subsequent experiments (Fig. 49).

(a) (b)

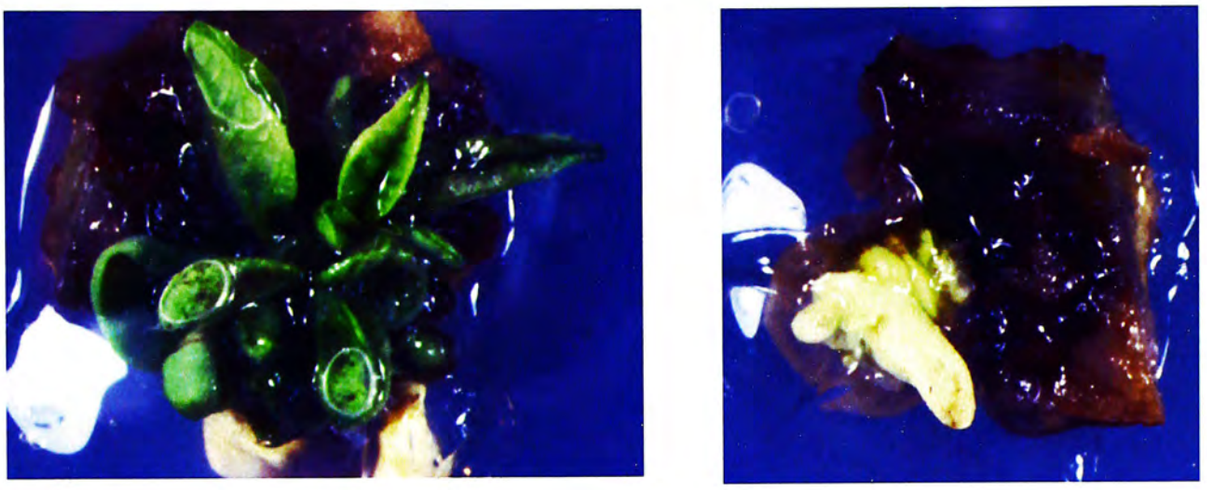
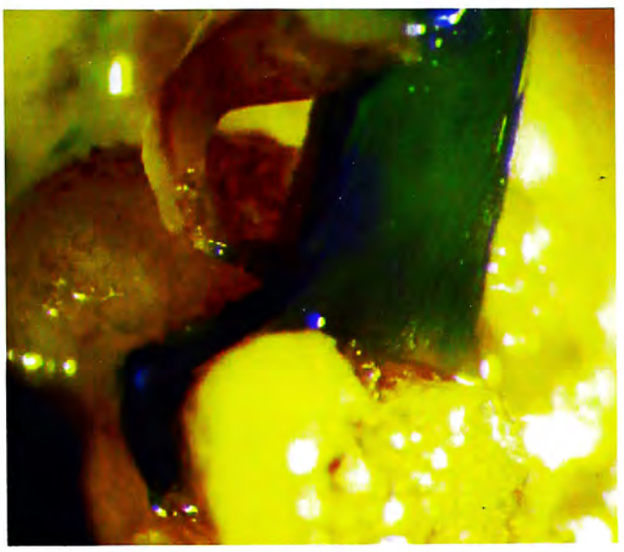
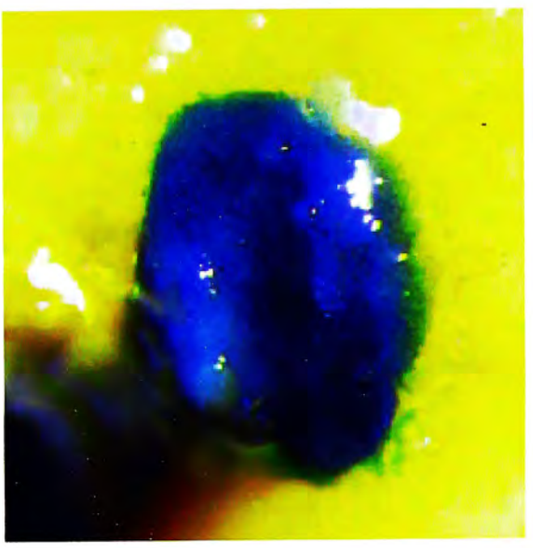


Fig. 48. Kanamycin selection of transformed soybean cotyledonary node explants. (a) Positive candidate appeared in green with multiple shoot. (b) Untransformed negative control that appeared in yellow and could not regenerate and differentiate into multiple shoots.

(a)



(b)



(c)

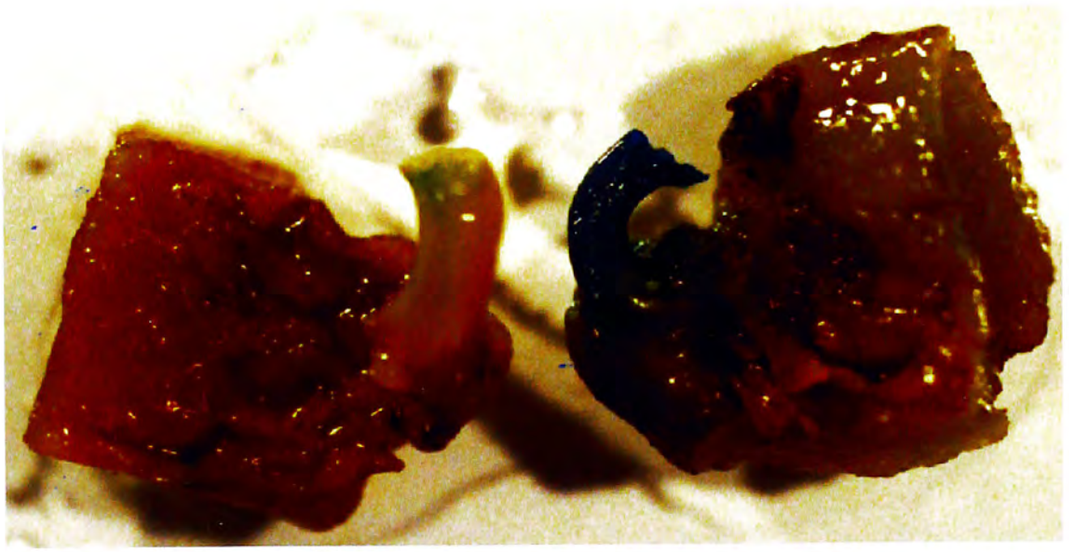


Fig. 49. GUS staining of different parts of the explants. Staining for GUS activities using X-gluc as the substrate was performed as described in the Materials and Methods Section. (a) Explant stained in blue at part of the hypocotyl and bud. (b) Amplified image of photo (a) at the regenerated bud region. (c) Positive and negative results of GUS staining, the explant on left was the negative candidate, whereas the one on right was considered as a positive transformant since its hypocotyl was stained blue.

3.3.2.2 Effects of plant hosts

A total of seven Chinese soybean varieties were tested. The five soybean varieties from Northern China (Ji lin xiao li no. 1, He feng no. 25, Ji lin no. 30, Ji lin no. 36 and Chang nong no. 5) were soybean transformation hosts currently employed in China (王連錚，王金陵，1992). However, these soybean varieties did not adapt well to the conditions in Southern China (i.e. Hong Kong) and hence two Southern varieties (Zhe chun no.3 and Ai jiao zao) were also tested. When comparing the germination frequency and regeneration efficiencies of the Southern varieties (Zhe chun no.3 and Ai jiao zao) with the commonly used Northern variety, Ji lin xiao li no. 1, Zhe chun no.3 and Ai jiao zao performed much better in Hong Kong (Table 20 and Table 21).

Kanamycin resistance assay and GUS staining assay were used to estimate the transformation efficiency of Northern varieties and Southern varieties, respectively. Comparison of the five Northern varieties was made (Table 22). After three preliminary experiments, explants prepared from Ji lin no. 36, He feng no. 25 and Chang nong no. 5 were found to exhibit lower germination efficiency and ability to survive. Further cultivars comparison was thus limited to Ji lin xiao li no. 1 and Ji lin no. 30. Ji lin xiao li no. 1 might exhibit the highest efficiency for *Agrobacterium*-mediated transformation. Transformation efficiencies of the two Southern varieties (Zhe chun no. 3 and Ai jiao zao) were compared with Ji lin xiao li no. 1 in a separated experiment. It was found that the transformation efficiencies of the two Southern varieties seemed to be even higher than that of Ji lin xiao li no. 1 (Table 23).

Table 20 Germination frequencies of Zhe chun no. 3, Ai jiao zao and Ji lin xiao li no. 1.

Soybean variety	No. of seeds sown	No. of seeds germinated	Germination efficiency (%)
Zhe chun no. 3	455	235	51.6
Ai jiao zao	455	230	50.5
Ji lin xiao li no. 1	565	140	24.8

Table 21 Regeneration efficiencies of Zhe chun no. 3, Ai jiao zao and Ji lin xiao li no. 1.

Soybean variety	Total number of explants	Number of explants with shoots	Regeneration efficiency (%)
Zhe chun no. 3	278	106	38.1
Ai jiao zao	266	86	32.3
Ji lin xiao li no. 1	106	10	9.4

Table 22 Comparison of transformation efficiencies among the five Chinese soybean cultivars.

Soybean varieties	GV 3101		Transformation efficiency (%)
	Total no. of explants	Positive explants[a]	
Ji lin xiao li no. 1	810	11	1.4
He feng no. 25	132	0	0.0
Ji lin no. 30	237	2	0.8
Ji lin no. 36	52	0	0.0
Chang nong no. 5	90	0	0.0

[a]The explants that could regenerate and differentiate into multiple shoot in kanamycin selective medium was scored as positive.

Table 23 Comparison of transformation efficiencies among Zhe chun no. 3, Ai jiao zao and Ji lin xiao li no. 1.

Soybean variety	**Total number of explants**	**Number of explant stained in blue in GUS staining**	**Transformation efficiency (%)[a]**
Zhe chun no. 3	108	32	29.6
Ai jiao zao	79	18	22.8
Ji lin xiao li no. 1	44	6	13.6

[a]Explants showing any GUS activities in regenerated bud, entire or part of hypocotyls were scored as positive.

3.3.2.3 Effects of *Agrobacterium* strains

As different *Agrobacterium* strains may exhibit differential virulence to different plant hosts. Two different commonly used *Agrobacterium* strains available in this laboratory (GV3101/ pMP90 and LBA4404/ pAL4404) were tested. Both strains were transformed with the plasmid pBI121, carrying a kanamycin resistance marker gene and a GUS reporter gene. Ji lin xiao li no. 1 was used as the plant host for transformation. A total of 600 seeds were used in this experiment. Transformation efficiency was estimated by counting the number of kanamycin resistant plantlets in the selective differentiation medium (Table 24). GV3101/ pMP90 was found to be more virulent to Ji lin xiao li no. 1 than LBA4404.

Table 24 Differential transformation efficiencies of Ji lin xiao li no. 1 for *Agrobacterium* strains, GV3101 and LBA4404.

***Agrobacterium* strains**	**Total no. of explants**	**Positive explants**	**Transformation efficiency (%)[a]**
GV3101	458	8	1.8
LBA4404	323	2	0.6

[a]The explants that could regenerate and differentiate into multiple shoots in kanamycin selective medium were scored as positives.

3.3.2.4 The application of vacuum infiltration

Vacuum infiltration, which is believed to bring close contact between plant cells and *Agrobacterium*, has been successfully used in *in planta* *Agrobacterium*-mediated transformation of flowering buds in *Arabidopsis thaliana* (Bechtold, *et al.*, 1993). A total of 250 seeds of Ji lin xiao li no. 1 were transformed with the *Agrobacterium* GV3101/ pMP90 carrying pBI121. The cotyledonary node explants were prepared as described in the Materials and Methods Section. For each pair of explants prepared form a cotyledonary node, one explant was vacuum infiltrated by co-cultivating the explants in *Agrobacterium* strain and applying vacuum for the remaining one, which was co-cultivated with the *Agrobacterium* strains without vacuum application, was used as the control.

The result of applying vacuum infiltration was showed in Table 25. Transformation efficiency was estimated as the number of regenerated explants in

kanamycin selective medium over the total explants used for transformation. The estimated transformation efficiency of using vacuum infiltration was comparatively lower than that of the control. This unexpected result might be due to physical damages on the delicate explants by vacuum suction. The apparent reduction of transformation efficiency was likely occurred at the regeneration step (i.e. vacuum treatment caused damages and inhibited regeneration).

Table 25 Application of vacuum infiltration during *Agrobacterium*-mediated transformation of soybean cotyledonary node.

	Total no. of explants	**Explants with shoot**	**Transformation efficiency (%)[a]**
Vacuum application	170	0	0.0
Without vacuum application	183	3	0.9

[a]The explants that could regenerate and differentiate into multiple shoots in kanamycin selective medium were scored as positives.

3.3.2.5 Effect of kanamycin

Most protocols for soybean transformation, the selective antibiotic kanamycin was added in the differentiation medium to screen for positive transformants when the kanamycin resistance gene was used as the selective medium. However, kanamycin may have negative effects on plant regeneration even in the cells containing the kanamycin resistance marker gene. To test this possibility, experiment was performed to test the harmful effect of kanamycin on explant regeneration. *Agrobacterium* strain, GV3101, and Ji lin xiao li no. 1 were used as experimental

materials. Explants regenerated in kanamycin selective medium were considered as positive candidates. For the no-kanamycin control, those regenerated into buds in differentiation medium without kanamycin and stained blue by GUS staining (Fig. 49; see also the Materials and Methods Section) were scored as positive transformants. The results were summarized in Table 26. In this experiment, the number of regenerated explants in differentiation medium without kanamycin was much higher than that containing kanamycin. Therefore, the inhibitory effects of kanamycin were likely at the plant regeneration step.

Table 26 Effects of kanamycin.

Differentiation medium	**Total number of explants**	**Number of positive candidates**	**Transformation efficiency (%)[a]**
With kanamycin	241	4	1.7
Without kanamycin	112	32	13.3

[a]For candidates grown on kanamycin containing medium, the explants that could regenerate and differentiate into multiple shoots in kanamycin selective medium were scored as positive. For candidates grown on medium without kanamycin, explants showing any GUS activities in regenerated buds, entire or parts of hypocotyls were scored as positive.

3.3.2.6 Effect of co-cultivation duration and light/ dark treatment during germination

The length of co-cultivation time between soybean explants and the *Agrobacterium* was tested to determine the optimal duration. Co-cultivation four or five days were studied. Besides, the effects of light/ dark treatment during

germination were also investigated. Ji lin xiao li no. 1 was used as the plant host and GV3101/ pMP90 *Agrobacterium* strain carrying pBI121 plasmid was employed to transform the soybean explants. The results were summarized in Table 27. In general, regeneration under light gave better result than that under dark conditions. The explants in the dark were found to be weak and hard to regenerate. In this particular plant-bacterium combination, a 5-day co-cultivation duration was better than a 4-day period. However, the exact transformation actually varied for different plant-bacterium combination.

Table 27 Effects of explant-bacterium co-cultivation duration and light/ dark treatment during germination.

Co-cultivation time	**Germinating under light/ dark**	**Total number of explants used**	**Explants with GUS positive foci**	**Transformation efficiency (%)[a]**
4	light	117	15	8.4
	dark	39	0	0.0
5	light	101	25	24.8
	dark	85	3	3.5

[a]Kanamycin was added in the differentiation medium. Explants that could regenerate and differentiate into multiple shoots in kanamycin selective medium were scored as positives.

3.3.3.6 Application of the detergent Silwet-77

The detergent Silwet-77 was reported to strongly increase the efficiency of *Agrobacterium*-mediated transformation of *A. thaliana* (Bechtold, *et al.*, 1993). It is believed that this detergent reduced the liquid surface tension of the suspension and

rendered the cell membrane of the explants more penetrable to the *Agrobacterium*. In this experiment, Ji lin xiao li no. 1 was used as the plant host for transformation of GV3101/ pMP90 and LBA4404/ pAL4404 cultures (both carrying pBI121) Regenerated buds appeared blue in the presence or absence of Silwet-77 using GUS staining were considered as positive candidates. The results were shown in Table 28, which suggested that the addition of Silwet-77 might raise the transformation efficiency only when GV3101/ pMP90 was used, but might not exhibit effects in the case of LBA4404/ pAL4404.

Table 28 Effect of the detergent Silwet-77.

Treatment	**Total number of explants**	**Explants with GUS positive foci**	**Transformation efficiency (%)[a]**
GV3101	39	11	28.2
GV3101 with detergent	37	17	46.0
LBA4404	34	6	17.7
LBA4404 with detergent	32	3	9.4

[a]Explants showing any GUS activities in regenerated buds, entire or part of hypocotyls were scored as positive.

3.3.5 Verification of transformation results by PCR screening

After obtaining the soybean transformation protocol according to the findings, further transformation was performed using the plant hosts Ai jiao zao, Zhe chun no.3 and Ji lin xiao li no.1 and the *Agrobacterium* strain, GV3101/ pMP90 (carrying pBI121 plasmid). PCR screening was done on regenerated plantlets being

differentiated leaves. Genomic DNA was extracted and PCR screened for the GUS gene (originated from pBI121) using oligos HMOL 703 and HMOL 704 (see the Materials and Methods Section). Typical positive results were shown in the Fig. 50. Four plantlets were shown to possess the GUS gene in their genomic DNA extracted from the older leaves (at lower part of the regenerated plantlets). However, DNA extracted from the younger leaves (upper part) from the same regenerated plantlets did not show a corresponding positive result. This controversy may be due to two possible reasons: (1) The regenerated plantlets are mixtures of transformed and untransformed shoots, older leaves that showing positive results might be raised from transformed cells, while the younger leaves giving negative results came from untransformed cell. (2) There were agrobacterial DNA contaminations in the samples.

To test the possibility of agrobacterial DNA contamination, PCR screening the agrobacterial gene *virA* was performed using the oligos HMOL 705 and HMOL 706 (see the Materials and Methods Section). Two out of the four positive candidates identified in Fig. 50 gave bands in size of about 1200bp indicating the presence of the *virA* gene and suggesting agrobacterial DNA contamination. However, the remaining two showing negative results containing that the GUS gene detected came from the soybean genomic DNA (Fig. 51).

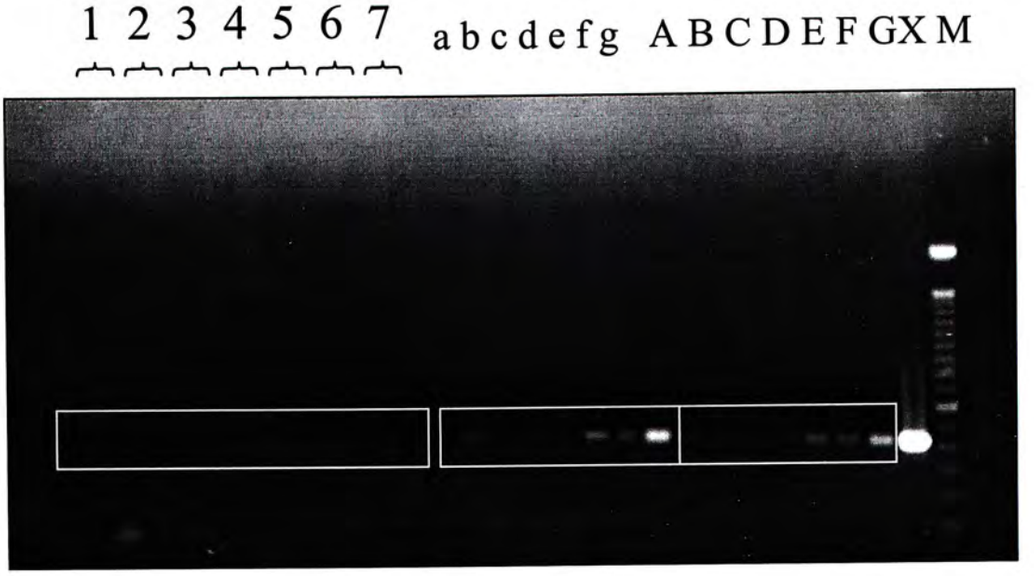


Fig. 50. PCR screening of GUS gene in transformed soybean. Lanes l 1, 2, 3, 4, 5, 6 and 7: PCR products of genomic DNA extracted from upper younger leaves, and each of the sample was duplicated; lanes a, b, c, d, e, f and g: duplicates of lanes A, B, C, D, E, F and G, respectively and were the PCR products of genomic DNA extracted from lower and older leaves; lanes X: positive control using the pBI121 plasmid DNA; and lane M: 1kb ladder (Gibco). The leaves used to extract genomic DNA were regenerated from Zhe Chun no.3. Samples A (a), E (e), F (f) and G (g) were considered as positive candidates.

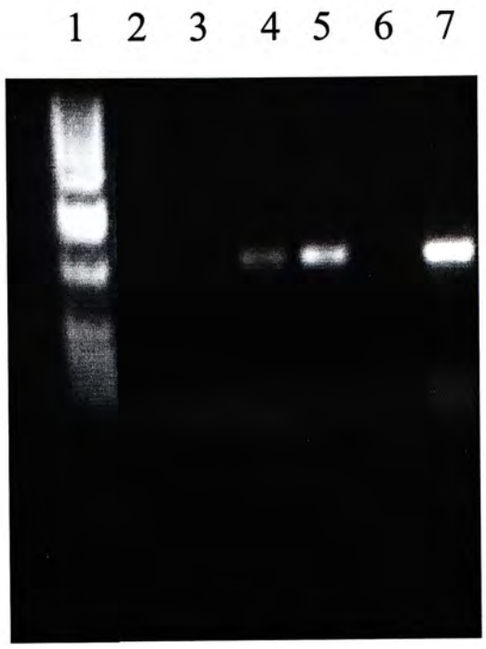


Fig. 51. PCR screening for the agrobacterial gene *virA*. Lane 1: 1kb ladder (Gibco); lanes 2-5: four samples identified as positive candidates in PCR screening of GUS gene (equal to A (a), E (e), F (f) and G (g) in Fig. 50, respectively); lane 6: negative control using genomic DNA extracted from untransformed Zhe Chun no. 3; and lane 7: positive control using pBI121 plasmid DNA.

Chapter 4 Discussion

As summarized in the literature review section, the combined genetic engineering and tissue culture techniques enables the construction of transgenic plants that exhibit: (1) alteration of endogenous metabolic pathways to producing (or not produce) the targeted metabolites; (2) expression of new genes to introduce desirable agronomic traits; and (3) expression of foreign genes to produce high-valued proteins. This research is to address the scientific questions posed in category (3).

Despite that there are several scientific papers claiming that transgenic plants can be used as bioreactors to generate pharmaceutical, agricultural and industrial proteins (see 1.1.2.2), very few (if any) real production lines were reported. The major obstacle lies in the functional and quantitative expression of a foreign protein in a transgenic plant.

In this research, experiments were performed to (1) test whether different categories (pharmaceutical and agricultural) of foreign genes can be expressed properly in transgenic plants, and (2) optimize a high output (high protein) transformation system (soybean).

To perform quick assay for the expression of transgenes, the model plant *A. thaliana* was used as the host. To prepare for large-scale product, transformation protocol for seed protein-rich soybean was investigated.

4.1 Expression of LCMV-NP, GHI and GHII in *A. thaliana*

LCMV-NP (pharmaceutical), GHI and GHII (agricultural) were chosen as the testing genes, based on their potential importance and availability.

The cDNA of LCMV-NP was obtained from Pasteur Institute via our collaborator Dr. M. F. Saron. Although the infection of LCMV to human is largely accidental (see 1.3.1.1) and there is no urgent need to produce vaccine against LCMV, this virus is an important model to study different types of immunogneic responses. Production of the antigen LCMV-NP in transgenic plant will allow the comparative studies of vaccine delivery via different routes (e.g. oral, intravenous injection, intraperitoneal injection, etc.).

Growth hormones, on the other hand, exhibit profound effects (probably via the downstream effectors such as the insulin-like growth factor) in controlling growth and development in animals (see 1.3.2.2). While goldfish growth hormone may induce cross reactivities to other fishes, more importantly, goldfish will allow easy functional assay of growth and thus may help to set up a production platform of using growth hormones to improve fish production.

The cDNAs encoding LCMV-NP, GHI and GHII were placed under the control of the CaMV 35S promoter (Kay, *et al.*, 1987; Gatz, *et al.*, 1991) to ecotopically express in plant cells. The *Agrobacterium* strain GV3101/ pMP90 and binary vector systems V7 and W104 have been successfully used to deliver targeted genes into the *A. thaliana* ecotype Col-0 via vacuum infiltration technique (Bechtold, *et al.*, 1993). Using this transformation platform, cDNAs of LCMV-NP, GHI and

GHII were all transformed into Col-0. A total of 9, 3 and 3 independent transgenic lines were obtained for LCMV-NP, GHI and GHII, respectively. Some of these transformants contained multiple inserts indicating that 2 or more T-DNA may integrate into the same plant cell genome occasionally. In most cases, transgenic lines containing only a single insertion locus were chosen for detailed studies. These lines are more stable genetically and are less susceptible to gene silencing due to the presence of multiple gene copies (Fagard and Vaucheret, 2000). Screening of lines containing single insertion locus was performed by scoring the kanamycin resistance (selection marker presents on the T-DNA) of T_2 progenies. Since the kanamycin resistance is a dominant character, a 3: 1 (resistant: sensitive) ratio will indicate a hemizygous status of a single insertion locus (Fig. 9, Table 14 and 17). Using Chi-square test with 0.05 significant level, 4, 3 and 3 independent transgenic lines were found to contain single insertion locus for LCMV-NP, GHI and GHII, respectively. To facilitate further analysis, homozygous lines of those single-insertion-locus were identified by 100% kanamycin resistance in their T_3 progenies.

Since kanamycin resistance only indicated the presence of the selection marker gene, Southern blot analysis was performed to verify the successful integration of the targeted transgenes. The data of Southern blot analysis not only confirmed positive transformation results, it also verified that the independently transformed lines carried single insert in different loci (Fig. 13 and Fig. 32).

After confirmation of the presence of transgenes in the plant genome, successful gene expression was tested by Northern blot analysis. For all LCMV-NP transgenic lines, a strong signal was observed when the cRNA of LCMV-NP clones

was used as probes (Fig. 14 and Fig. 15). The LCMV-NP cDNA thus has no problem to transcribe in transgenic *A. thaliana*.

On the other hand, GHI and GHII cRNA probes also gave positive hybridized signals with the RNA prepared from GHI and GHII transgenic lines (Fig. 33 and Fig. 34). While strong expression was observed in all GHI transgenic lines, heterogeneous level of expression were found in different GHII transgenic lines. This might be resulting from gene silencing at transcriptional level in the low expression lines.

Since these lines contain single insertion loci, gene silencing resulted from epistatic trans-inactivation is unlikely. For single insertion, gene silencing might be induced by several mechanisms. In some cases, transgene methylation might occur at homozygous state via paramutation (Mayer and Heidmann, 1994). Position effects of the insertion might also play a role. For instance, if the transgene was inserted in a hypermethylated region, it may gradually become methylated and the transcription would be reduced/ ceased (Mayer and Heidmann, 1994). Exactly how the cell recognizes foreign DNA is still unknown. One possible mechanism is to sense the uncoupled GC contents of insert to its neighboring sequence in specific isochors of the plant genome (Mayer and Heidmann, 1994). In addition, transcription activity may make the transgene a specific target for DNA methylation, since when promoter activity opens the local chromatin structure, cellular protein involved in DNA methylation may become accessible (Mayer and Heidmann, 1994).

The most critical part of this research is to study the product of the target proteins. Following the Northern blot analysis, recombinant protein production was

studied by radioimmunoassay (for GHI and GHII transgenic plants), western blot and protein dot blot analyses (for LCMV-NP transgenic plants).

For LCMV-NP transgenic plants, recombinant protein produced from bacterial system was used as positive control, while polyclonal antibody from mice against LCMV viral infection was adopted as the primary antibody. No positive result was obtained in the Western blot analysis (Fig. 26). To maximize the protein sample loaded for detection, protein dot blot experiment was performed. However, there was still no detectable level of LCMV-NP from the transgenic plants (Fig. 27).

For GHI and GHII transgenic lines, crude protein extracts were isolated from the homozygous transgenic lines Radioimmunoassay (RIA) was performed by Prof. A. O. L. Wong (The University of Hong Kong) (Fig. 36). Some transgenic lines showed low but clearly detectable levels of the recombinant GHI and GHII proteins (in range of 0.0004-0.003%). In this experiment, low salt buffer and high salt buffer were used to extract the soluble and cell-wall bound proteins, respectively. Although high salt buffer alone gave some background noise in RIA, positive signals were still easily distinguishable (Fig. 36). In GHII experiments, the mRNA levels did not show a positive correlate with the recombinant protein quantities. The protein levels were either too low to perform quantitative analysis or post-transcriptional gene silencing was taking place.

Since a problem was encountered at the protein synthesis level, *in vitro* transcription and translation using rabbit reticulocyte lysate (RRL) and wheat germ extract (WG) were performed to verify the transcriptional and translational compabilities of the target transgenes.

As illustrated in Fig. 45, all gene constructs can produce recombinant proteins of the expected sizes. GHII was produced at a lower level in both RRL and WG systems, when compared to GHI. For LCMV-NP, the recombinant protein gave a strong signal in wheat germ system, but only a weak band was observed when RRL system was used. Thus, all target constructs except GHII exhibits no major problems in using the plant protein expression machinery.

The low levels of recombinant protein product in the transgenic lines may be due to several reasons. At the transcriptional level, although signals were detected in Northern blot analyses, the absolute level may still be too low for quantitative production of recombinant protein. In general, the CaMV 35S promoter only give recombinant protein expression levels of 0.01-0.1% (Pen, *et al.*, 1992; Hiatt, *et al.*, 1989; Verwoerd, *et al.*, 1995). On the other hand, seed specific promoters can drive the expression of a transgene product up to a level of 8-10% of the total soluble proteins (Atlenbach, *et al.*, 1989 and Falco, *et al.*, 1995). In addition, translational fusion of GUS coding sequence with a consensus sequence (AACAATGG) around the translational start was reported to triple the GUS activity (Guerineau, *et al.*, 1992). This consensus sequence was not included to the gene constructs in this research.

Another possible problem is the occurrence of post-transcriptional gene silencing. It may result from the unexpected production of anti-sense RNA from converging promoter, within or near the T-DNA (Burd and Dreyfuss, 1994). The anti-sense RNA produced will hybridize with the sense mRNA generated by the transgene. In some cases, the double-stranded RNA will be act on by specific ribonucleases (Burd and Dreyfuss, 1994). However, it may not be applicable to this research (expect in some GHII transgenic lines), since the steady-state mRNA levels

of most transgenes did not seem to be affected.

The problem associated with those RNA hybrids may lie in the reduction of translation efficiency. The ribosome binding in double-stranded RNA may be impaired and thus decrease protein production. Moreover, RNA hybrid may be a preferential substrate for maintenance methylase that will methylate the sense mRNA and impair translation accordingly (Burd and Dreyfuss, 1994).

Besides, ineffective RNA transport (for RNA hybrid or the methylated sense mRNA of the transgenes) may also play a role in post-transcriptional gene silencing. Transport of mRNA from nucleus to cytoplasm involves RNA-protein complex. This process is energy-dependent and is saturable by increased concentrated of RNAs. Transport of different RNA species requires specific protein factors. In addition, the RNA binding proteins exhibit different affinities towards different RNA sequences. Thus, gene silencing can be the result of shortage of this transport protein. Alternatively, dose-dependent control may also play a role at the cytoplasmic level, where saturable protein factor is involved in stabilization of mRNA transcript of the transgenes (Burd and Dreyfuss, 1994).

Another commonly encountered factor affecting translational efficiency is codon usage. Since enhanced tRNA composition was used in the *in vitro* translation experiments, the effects of differences in codon usages could not be verified. *A. thaliana* has its own preference of codon usage and the composition of t-RNA pools might not couple with the need of the transgene. Differences of codon usages in GHI, GHII and LCMV-NP to *A. thaliana* were found (Table 29).

Table 29 Codon usages in *A. thaliana*, LCMV-NP, GHI and GHII.

Amino acids	**Codon**	***A. thaliana* (%)[a]**	**LCMV-NP (%)[a]**	**GHI (%)[a]**	**GHII (%)[a]**
Phe	UUU	2.25 (52.08)	1.52 (50.00)	1.90 (40.00)	1.90 (44.44)
	UUC	2.07 (47.92)	1.52 (50.00)	2.80 (60.00)	2.37 (55.56)
Ser	UCU	2.46 (28.21)	1.25 (15.56)	1.90 (19.05)	1.90 (20.00)
	UCC	1.08 (12.39)	0.89 (4.44)	1.90 (19.05)	1.90 (20.00)
	UCA	1.78 (20.41)	2.50 (31.11)	0.95 (9.52)	0.95 (10.00)
	UCG	0.89 (10.21)	0.54 (6.66)	0.47 (4.76)	0.95 (5.00)
	AGU	1.40 (16.06)	0.89 (11.11)	1.42 (14.29)	1.42 (15.0)
	AGC	1.11 (12.73)	1.97 (24.44)	3.32 (33.33)	2.84 (30.00)
Tyr	UAU	1.52 (52.60)	1.25 (58.33)	0.00 (0.00)	0.00 (0.00)
	UAC	1.37 (47.40)	0.89 (41.67)	1.42 (100.00)	1.90 (100.00)
Cys	UGU	1.08 (60.00)	0.89 (71.43)	0.47 (20.00)	0.95 (50.00)
	UGC	0.72 (40.00)	0.36 (28.57)	1.90 (80.00)	0.95 (50.00)
Leu	CUU	2.43 (25.85)	2.86 (25.39)	1.90 (12.50)	0.47 (3.13)
	CUC	1.59 (16.91)	2.68 (23.81)	1.42 (9.38)	2.84 (18.75)
	CUA	1.00 (10.64)	1.07 (9.52)	0.00 (0.00)	0.47 (3.13)
	CUG	**0.99 (10.53)**	**1.61 (14.29)**	**7.11 (46.88)**	**7.58 (50.00)**
	UUA	1.29 (13.72)	0.89 (7.94)	0.95 (6.25)	0.47 (3.13)
	UUG	2.10 (22.34)	2.15 (19.05)	3.79 (25.00)	3.32 (21.88)
Pro	CCU	1.83 (38.33)	1.07 (23.08)	0.95 (25.00)	1.42 (37.50)
	CCC	1.53 (11.04)	0.89 (19.23)	1.90 (50.00)	1.42 (37.50)
	CCA	1.61 (38.33)	2.15 (46.15)	0.47 (12.25)	0.95 (25.00)
	CCG	0.83 (17.29)	0.54 (11.54)	0.47 (12.25)	0.00 (0.00)
His	CAU	1.40 (61.67)	0.54 (37.50)	0.47 (33.33)	0.00 (0.00)
	CAC	**0.87 (38.33)**	**0.89 (62.50)**	**0.95 (66.67)**	**1.42 (100.00)**
Arg	AGA	1.87 (35.15)	2.50 (50.00)	2.37 (38.46)	2.37 (38.46)
	AGG	1.09 (20.49)	2.50 (50.00)	0.95 (15.38)	0.95 (15.38)
	CGU	0.89 (16.73)	0.00 (0.00)	0.95 (15.38)	0.95 (15.38)
	CGC	0.37 (6.95)	0.00 (0.00)	1.42 (23.08)	1.42 (23.08)
	CGA	0.62 (11.65)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	CGG	0.48 (9.02)	0.00 (0.00)	0.47 (7.69)	0.47 (7.69)
Ile	AUU	2.20 (41.20)	2.33 (43.33)	0.95 (25.00)	2.84 (60.00)

	AUC	1.85 (34.64)	1.97 (36.67)	2.84 (75.00)	1.42 (30.00)
	AUA	1.29 (24.16)	1.07 (20.00)	0.00 (0.00)	0.47 (10.00)
Thr	ACU	1.78 (34.50)	1.07 (24.00)	0.95 (22.22)	0.95 (22.22)
	ACC	1.03 (19.96)	0.72 (16.00)	2.84 (66.67)	2.84 (66.67)
	ACA	1.59 (30.81)	2.50 (56.00)	0.47 (11.11)	0.47 (11.11)
	ACG	0.76 (14.73)	0.18 (4.00)	0.00 (0.00)	0.00 (0.00)
Met	AUG	2.45 (100.00)	3.22 (100.00)	3.32 (100.00)	2.84 (100.00)
Asn	AAU	2.27 (52.06)	2.15 (44.44)	1.90 (28.57)	2.37 (31.25)
	AAC	2.09 (47.94)	2.68 (55.56)	4.74 (71.43)	5.21 (68.75)
Lys	AAA	3.10 (48.47)	2.86 (37.21)	1.90 (40.00)	1.90 (36.36)
	AAG	3.26 (51.26)	4.83 (62.79)	2.84 (60.00)	3.32 (63.64)
Gln	CAA	1.97 (56.45)	2.33 (39.39)	0.95 (20.00)	0.95 (22.22)
	CAG	1.52 (43.55)	3.58 (60.61)	3.79 (80.00)	3.32 (77.78)
Trp	UGG	1.27 (100.00)	1.25 (100.00)	0.47 (100.00)	0.47 (100.00)
Val	GUU	2.73 (40.38)	3.04 (44.74)	0.95 (15.38)	0.95 (18.18)
	GUC	1.27 (18.79)	0.89 (13.16)	2.37 (38.46)	1.42 (27.27)
	GUA	1.01 (14.94)	0.89 (13.16)	0.00 (0.00)	0.47 (9.09)
	GUG	1.75 (25.89)	1.97 (28.95)	2.84 (46.15)	2.37 (45.45)
Ala	GCU	2.80 (43.28)	1.79 (28.57)	1.42 (27.27)	1.42 (27.27)
	GCC	1.03 (15.92)	1.25 (20.00)	0.47 (9.09)	1.42 (27.27)
	GCA	1.76 (27.20)	2.50 (40.00)	2.84 (54.55)	1.42 (27.27)
	GCG	0.88 (13.60)	0.72 (11.43)	0.47 (9.09)	0.95 (18.18)
Asp	GAU	3.60 (67.54)	2.68 (38.46)	2.84 (46.15)	2.84 (46.15)
	GAC	**1.73 (32.46)**	**4.29 (61.54)**	**3.32 (53.85)**	**3.32 (53.85)**
Glu	GAA	3.44 (51.65)	3.04 (68.00)	1.42 (25.00)	0.95 (16.67)
	GAG	3.22 (48.35)	1.43 (32.00)	4.27 (75.00)	4.74 (83.33)
Gly	GGU	2.22 (34.00)	1.43 (22.86)	0.47 (14.29)	0.47 (12.50)
	GGC	0.90 (13.78)	1.43 (22.86)	0.95 (28.57)	0.47 (12.50)
	GGA	2.39 (36.60)	0.89 (14.28)	0.47 (14.29)	1.42 (37.50)
	GGG	1.02 (15.62)	2.50 (40.00)	1.42 (42.86)	1.42 (37.50)

[a]% frequency of the corresponding codons usage calculated based on known sequences of the whole genome (*A. thaliana*) or individual genes (LCMV-NP, GHI and GHII). Figures in parenthesis indicate the relative % frequency of codon usage for each individual amino acid. Possible differences of codon usage were highlighted in bold.

After *in vivo* synthesis systems of the recombinant proteins, the next problem lies in their stability. Most proteases found in vegetative organs of plants are cysteine-type endoproteases that are localized in vacuole. Besides, ubiquitin-mediated general protein degradation pathway mainly occurs in cytoplasm. As the recombinant proteins were not targeted to any subcellular location after translation, they should remain in cytoplasm and may become targets for ubiquitination. To tackle this problem, fusing a peptidic signal to the recombinant protein to direct its secretion to extracellular compartment or retent it in endoplasmic reticulum would be helpful to protect the target protein from degradation. For instance, fusing tetrapeptidic signal Lys-Asp-Glu-Leu (KDEL) to pea vacuolar storage protein vicilin carboxy-terminal end increased its accumulation in transgenic mesophyll cells of *Medicago sativa* (alfalfa) by 100-fold (Wandelt, *et al.*, 1992).

In general, the N-terminal sequence of a protein will influence its stability in the cell. It is termed the N-end rule. Certain amino acids (e.g. Met, Thr, Ser, Gly or Val) at the N-terminal of protein will confer stabilizing effect to the proteins in cytosol. However, when amino acids such as Lys, Arg, His, Phe, Tyr, Trp, Ile, Leu, Asp, glu, Gln or Asn are present instead, the proteins are rapidly degraded by the ubiquitin system in the cytoplasm and nucleus (Varshavsky, 1997). Although all proteins are initiated with Met, the N-terminal Met could be removed by an aminopeptidase to expose the second amino acid, which then becomes the new N terminus of the protein. All the three targeted genes (GHI, GHII and LCMV-NP) initiated with methionine, and with Ser, Ser and Ala as the second amino acid, respectively. Serine and alanine were neither classified as stabilizing amino acids nor destabilizing amino acids, so no definitive conclusion can be drawn. In future studies, replacing the second amino acid to a stabilizing one may help to enhance the stability

of these recombinant proteins.

4.2 Establishing a soybean transformation system

Despite that the United States is the largest exporter of soybean products, soybean is not a native species in Northern American. Therefore, only a few cultivars are available in the Unitcd States and this gene pool is very narrow. Modification of soybean via genetic engineering hence would be imported for the development of new varieties for consumption. In this project, on the other hand, soybean is targeted due to its high contents of seed proteins. Genetically modified soybean is expected to give high output of high-valued recombinant proteins.

Since the first reports of soybean transformation (Hinchee, *et al.*, 1988; Kung and Wu, 1993), only a handful of laboratories have had more than sporadic success. The major methods for soybean transformation were confined to *Agrobacterium*-mediated transformation of cotyledonary nodes, particle bombardment of shoot meristems or embryogenic suspension culture and protoplast transformation. Comparing these transformation methods, *Agrobacterium*-mediated transformation of cotyledonary nodes offers the best platform for regeneration of fertile soybean plants since there is no requirement for the maintenance of parental donor plants (for providing leaf tissue or floral bud) or long-term cell cultures.

The efficiency of *Agrobacterium*-mediated gene transfer into the plant host genome is actually a function integrating the complex signal exchanges between the *Agrobacterium* and the plant host. If explants are used as the transformation materials, the efficiency of plant regeneration is another crucial factor. Lastly, an

effective screening system is also critical for identifying the successful transformation.

To optimize the *Agrobacterium*-mediated transformation system for soybean, systematic analysis of various factors affecting the transformation and regeneration of soybean cotyledonary node explants was performed in this study. For easy assay of the transformation with *Agrobacterium* containing the pBI121 plasmid. A kanamycin resistance gene and a GUS reporter gene are both present in the T-DNA.

4.2.1 Plant hosts and explants

As described by Owen and Cress, there was genotypic variation of soybean cultivars in response to *Agrobacterium* strains. Only 2.5% of nearly 1000 soybean cultivars were susceptible to *Agrobacterium* (Owen and Pen, 1996). A genetic basis for susceptibility has been noted in soybean (Mauro, *et al.*, 1995).

As expected, different soybean cultivars exhibited different efficiencies towards *Agrobacterium*-mediated transformation (Table 22 and 23). In this research, the adaptation capability of different cultivars to a local region was also considered. While the Northern cultivar (Ji lin xiao li no. 1, He feng no. 25, Ji lin no. 30, Ji lin no. 36 and Chang nong no. 5) showed reasonable transformation efficiency (estimated by transient assays; see Results), the overall performance of the Southern cultivars Zhe chun no. 3 and Ai jiao zao were much better (experiments performed in Hong Kong; Southern part of China) (Table 23).

The choice of a right explant tissue that is competent for both transformation

and regeneration is a crucial step both forward. Some reports suggested that only dedifferentiating cells were competent for transformation. The cells are commonly small, isodiametric and had prominent nuclei and a dense cytoplasm (Sangwan, *et al.*, 1992). These cells includes subepidemal cells near the vascular system of cotyledon and epicotyl regions, dedifferentiating mesophyll cells of leaf tissues and dedifferentiating pericycle of root tissues (De Kathen and Jacobsen, 1995). Thus, cotyledonary node was chosen as explants for transformation because it was the tissue both transformation-competent and regeneration-competent. Data of this research in general confined this (Table 21, 22 and 23; Fig. 46 and 47).

4.2.2 Regeneration of explants

Using the protocol presented in the Materials and Methods Section, the regeneration frequency is generally high. Nine to thirty-three percent of successful regeneration was resulted from soybean cotyledonary node explants of three soybean cultivars including Zhe chun no. 3, Ai jiao zao and Ji lin xiao li no. 1 (Table 21).

However, during the actual transformation process, additional factors may also affect the regeneration efficiency. One factor is due to the negative effect of *Agrobacterium* on the explants during and after the co-cultivation step. In theory, a longer co-cultivation between the explants and *Agrobacterium* should allow a high probability of T-DNA transfer. However, prolonged co-cultivation under conditions unfavourable for the growth of explants (e.g. grow in dark) will retard regeneration (Table 27). The optimal period for co-cultivation actually varied among different batches of seeds, depending on the seed quality. After co-cultivation, the *Agrobacterium* must be removed by applying antibiotics (see the Materials and

Methods Section). Otherwise, the *Agrobacterium* will overgrow and impede explant regeneration.

Another hindrance factor is due to the applying of selection agent. Kanamycin resistance is a commonly used selection marker that is also included on the T-DNA of pBI121 (our testing construct).

In this research, it was found that the number of regenerated explants in differentiation medium without kanamycin was much higher than that with kanamycin (Table 26). More importantly, the number of explants showing positive foci using GUS staining was much higher, when compared to the number of explants considered as positive transformants growing in kanamycin supplemented medium. This reflected that the actual transformation incidence could be more frequent, but yet hidden by the low regenerating ability of the explants growing in the presence of kanamycin. The transformed cells are generally surrounded by untransformed cells in which the growth is severely inhibited by kanamycin. These untransformed cells may secrete toxic metabolites or affect nutrient transportation efficiency. The transformed cells thus may not be able to regenerate and differentiate, even though it can resist to the selective agent (Norelli and Aldwinckle, 1993).

Although the elimination of kanamycin could enhance the regeneration of transformed explants, the absence of selection also associates with some disadvantages. Firstly, the system becomes more expensive and labor-intensive, as it involves the use of large amount of GUS staining reagents and the setting up of an extensive PCR screening scheme. Moreover, as all meristematic cells, including the untransformed cells, are allowed to regenerate and differentiate, chimerism may

occur.

4.2.3 *Agrobacterium* strains

Agrobacterium, the indispensable tool used in plant transformation, behaves differently among different strains. Byrne and co-workers evaluated eleven *Agrobacterium* strains on the most susceptible soybean genotype found, Peking, and found significant differences in tumor formation induced by different *Agrobacterium tumefaciens* strains (Byrne, *et al.*, 1987).

Two commonly used *Agrobacterium* strains GV3101/ pMP90 and LBA4404/ pAL4404 (see the Materials and Methods Section) were available in this laboratory. To select the strain more virulent to soybean, their transformation efficiencies were compared using Ji lin xiao li no. 1 as the soybean host. The results suggested that GV3101/ pMP90 may be more effective in transforming soybean, at least in the cultivar Ji lin xiao li no. 1 (Table 24).

4.2.4 Bacteria-plant interaction

The success of *Agrobacterium*-mediated plant transformation depends ultimately on the interaction between the bacteria and the plant host. Before T-DNA transfer can be taken place, the bacteria must be brought to a proximity to the plant cells. A commonly used strategy is wounding. In this research, wounds were introduced at the cotyledonary node, the region that is both transformation-competent and regeneration-competent. In addition to the removal of physical barriers between plant cells and bacterial cells, wounding of plant tissues also induce the secretion of

phenolic compounds that are essential in establishing the *Agrobacterium* infection process and the *vir* genes for T-DNA transfer (Charles, *et al.*, 1992; Hooykaas and Beijersbergen, 1994). Although some reports suggest that *Agrobacterium* can infect plant cells without wounding sites, the efficiency was comparatively lower than those with wounding (Escudero and Hohn, 1997).

Physical and chemical means may be employed to enhance the accessibility of *Agrobacterium* to the plant cells. Similar to the Sonication Assisted *Agrobacterium*-mediated Transformation (SAAT), vacuum infiltration was reported to enhance soybean transformation (Trick, *et al.*, 1997) by forcing *Agrobacterium* into wounded tissues which were macerated before vacuum application. However, as Harold and co-workers pointed out, the strength of vacuum applied should be determined empirically for each tissue (Santarem, *et al.,* 1998). Thus, modifications and optimizations were required for each transformation system using different plant hosts and *Agrobacterium* strains. For example, mannitol could be included in the medium for co-cultivation during vacuum application, since it could act to protect the target tissue from wounding during SAAT (Santarem, *et al.,* 1998).

However, when vacuum was applied to the cotyledonary node explants in this research, a negative result was observed (Table 25). Since all explants turned brown after the vacuum application, physical damages may have been taken place. The apparent transformation efficiency, which is dependent on both regeneration and T-DNA transfer efficiency, is thus reduced.

Osmotic salt was reported to have a positive effect on particle bombardment-mediated transformation of soybean and maize suspension culture

(Finer and McMullen, 1991). Therefore, the addition of mannitol to explant preparatory medium might also increase the accessibility of *Agrobacterium* to explant tissues. Since the resuspension medium of *Agrobacterium* exhibited comparable higher osmotic potential than the explant preparatory medium, *Agrobacterium* together with water would rush into the explants when the explants were transferred into the *Agrobacterium* suspension. Mannitol was thus included in the resuspension medium used in this research (see the Materials and Methods Section).

Another measure used to enhance the accessibility of *Agrobacterium* to explant was the application of detergent such as Silwet-77. Silwet-77 added in the *Agrobacterium* resuspension medium may soften the cell membrane of explants and reduce surface tension of explants and thus enhance the entry of bacteria into the relatively inaccessible plant tissues (Clough and Bent, 1998).

In Table 28, it was shown that the addition of Silwet-77 would greatly increase the transformation efficiency of GV3101/ pMP90 but decrease that in LBA4404/ pAL4404. LBA4404/ pAL4404 may be hypersensitive to the detergent Silwet-77.

How the bacteria and plant cells interact during the co-cultivation period is the next question to be addressed.

Although transgenic plants obtained via *Agrobacterium*-mediated method commonly used a 2-day co-cultivation period, longer co-cultivation time of 3 or 4 days can improved the transformation efficiency. De Bondt and co-workers reported

that a 4-day co-cultivation of *Malus* explants with *Agrobacterium* could be used but that bacterial overgrowth might become problematic with this longer co-cultivation period. For soybean transformation, Santarem and co-workers investigated the effects of the co-cultivation duration on transient GUS gene expression in immature cotyledons of soybean. It was found that co-cultivation time was positively correlated with the level of transient GUS expression (Santarem, *et al.*, 1998).

In this research, the optimal period for co-cultivation was found to vary depending on quality. In general, the explants became too weak to regenerate after a prolonged co-cultivation period in the dark (Table 27). Germination under light, on the other hand, tended to give better result (Table 27).

During the co-cultivation period, T-DNA transfer will be taken place and this process depends on both the state of bacteria and plant cells. As reviewed in the Literature Review Section, phenolic compounds released from wounds are responsible for activity of the bacterial *vir* genes that in turn control the T-DNA transfer (Delzer, *et al.*, 1990; Stachel, *et al.*, 1985; Trick and Finer, 1997).

Besides, co-cultivation in the presence of phytohormones was reported to increase transformation efficiency (Bergmann and Stomp, 1992; Lowe and Krul, 1991; Sangwan, *et al.*, 1992). The phytohormones may induce active division of explant cells that was reported to be desirable for T-DNA integration (Villemont, *et al.*, 1997).

Both the phenolic compounds acetosyringone and phytohormones were thus included in the co-cultivation medium to enhance transformation efficiency.

4.2.5 Transient versus stable transformation

Since kanamycin imposes a negative effect on explant regeneration, all regenerated shoots must be subjected to further screening using techniques such as PCR.

Several hundreds cotyledonary nodes were used and nearly hundred explants regenerated and differentiated into small plantlets. Genomic DNA was extracted from their leaves and PCR screening for GUS gene was performed. Four plantlets were shown to possess the GUS gene in genomic DNA extracted from lower and older leaves. However, DNA extracted from the upper leaves from the same regenerated plantlets did not show positive result (Fig. 50). There were two possibilities: (1) the plantlets are mixtures of transformed and untransformed shoots, i.e. the lower leaf that showed positive result might be raised from a transformed cell, while the upper leaf giving negative result came from an untransformed cell; (2) there was agrobacterial DNA contamination.

In order to test the possibility of agrobacterial DNA contamination, PCR screening of the bacterial *virA* gene was performed using the oligos HMOL 705 and HMOL 706 (Fig. 51). Two out of the four candidates gave bands in size of around 1200bp which were probably part of the *virA* gene. However, the remaining two exhibited negative result, suggesting that the GUS gene was come from the soybean.

Therefore, these two plantlets might be shoots mixtures, in which only some shoots were transformed. Besides, since all the upper and younger leaves showed negative results, untransformed meristematic tissue might have a higher regeneration

and differentiation capability and become dominant in the plantlets. As mentioned in section 4.3.4, the formation of shoot mixture will be enhanced when kanamycin is not included in the differentiation medium, since all meristematic cells including transformed and untransformed ones are allowed to regenerate. To obtain stable transgenic soybean, the shoot mixtures have to be transferred to soil, selfed and allowed to give progenies. If the transformed cells contributed to germ-line cells, transgenic progenies could be obtained. For the problem due to the overgrowth of the untransformed tissue in the explants, subculturing might be one of the solutions. Another alternative is to explore other non-inhibitory selective agents that can enrich transformed tissue but give least harm on plant regeneration. For example, glucuronide derivative of cytokinin benzyladenine can be used as selective agent. The inactive cytokinin, upon hydrolysis by GUS, will be converted to cytokinin and stimulate the transformed cell to regenerate (Joersbo and Okkels, 1996).

It is worth to note that the relatively small number of PCR-positive transformants (Fig. 50) is not consistent with the high frequency of GUS-positive foci formation in transformed cotyledonary node explants when tested with GUS staining assays (Table 26). This phenomenon is consistent with the view that many species are susceptible to transient transformation, but are also highly recalcitrant to stable transformation (Gelvin, 2000). Recently, the problem can be partially overcome by overexpressing plant genes involved in T-DNA integration (e.g. *A. thaliana* histone H2A gene, *RAT5* (Mysore, *et al.*, 2000). This new feature should be incorporate into the protocol for further optimization of the soybean transformaton system.

4.3 Conclusion and perspective

This research addressed to the feasibility of using transgenic plants as bioreactors to produce high-valued proteins. Two plant hosts were used for the transformation. The model plant, *Arabidopsis thaliana*, was used for quick assays, while the protein-rich *Glycine max* (soybean) was used for large-scale production. To test the production scheme, two categories (pharmaceutical and agricultural) of products were examined.

LCMV-NP, GHI and GHII transgenic *A. thaliana* were constructed. The presence and expression of the transgenes were confirmed by Southern and Northern blot analyses (except some transgenic lines for GHII). However, recombinant proteins could not be detected (for LCMV-NP via Western blot and protein dot blot analyses) or only be detected in low levels (for GHI and GHII by mean of RIA). *In vitro* translation approach, on the other hand, confirms that the recombinant DNA can indeed generate protein products of the expected sizes.

For the establishment of protein-rich soybean transformation system, *Agrobacterium*-mediated transformation system using cotyledon nodes as explants was adopted. Seven Chinese soybean cultivars and two *Agrobacterium* strains were tested. Several optimizations of the transformation protocol were made so as to enhance the transformation and regeneration efficiencies. Transient expression of GUS reporter gene (presented in the T-DNA) was confirmed at the regenerated buds. However, the incidence of stable transformation of the reporter gene was low and formation of shoot mixtures was observed. Moreover, the growth of stable transformed tissues was retarded by untransformed tissues in the shoot mixtures.

As recombinant proteins could not be detected or only be detected in low levels, modifications to the constructs are required. Stronger promoter, for example, seed-specific promoter (phaseolin promoter) could be used instead of CaMV 35S promoter. Besides, fusing the targeted genes with a signal sequence (e.g. sequence encoding KDEL) could bring the recombinant proteins to compartments (e.g. endoplasmic reticulum) that have few proteases. In addition, basing on the N-end rule, modifications of the second amino acid of the recombinant protein to a stabilizing one may help to protect them from ubiquitination. Another potential improvement is to optimize the codon usages of target genes to match that of the plant host.

For soybean transformation system, subculturing of transformed plantlets could help to reduce shoot mixture formation. Furthermore, co-transformation of plant genes (e.g. *RAT5*) involved in T-DNA integration with the target genes may increase the stable transformation frequency.

Despite the difficulty in increasing the yield of recombinant proteins in transgenic plants, the potential of producing high-valued proteins using plant as a bioreactor is enormous because of the relative ease of scale-up and decreased risk of animal pathogen contamination when compared to conventional production platform.

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Appendix I – Restriction and modifying enzymes:

1. *BamH*I	Promega R6021
2. *Bbu*I	Promega R6621
3. *Bgl*II	Promega R6081
4. *Dra*I	Promega R6271
5. *EcoR*I	Promega R6011
6. *EcoR*V	Promega R6351
7. *Hind*III	Promega R6041
8. *Nde*I	NEB 111S
9. *Pst*I	Promega R6111
10. *Sac*I	Promega R6061
11. *Sal*I	Promega R6051
12. *Sma*I	Promega R6121
13. T3 RNA polymerase	Promega P2083
14. T4 DNA ligase	Promega M1804
15. T7 RNA polymerase	Promega P2073
16. Taq DNA polymerase	Boehringer 1647679
17. *Xba*I	Promega R6181
18. *Xho*I	Promega R6161

Appendix II – Chemicals:

1. Acetosyringone	Aldrich Chem D134406
2. Acrylamide/ Bis	Bio-Rad 161-0120
3. Ammonium acetate	Ajax 27
4. Ammonium persulfate	Bio-Rad 161-0700
5. Ampicillin	Sigma A9518
6. Agarose	GibcoBRL 15510-027
7. Ascorbic acid	Sigma A7506
8. Bacto-peptone	Difco 0118-01-8
9. Bacto™ Agar	Difco 214010
10. Benzyl-aminopurine	Sigma B5898
11. Bis	Sigma M7279
12. Blocking reagent	Boehringer 1096176
13. Boric acid	Ajax 101
14. Bovine serum albumin	Sigma A7906
15. Bromophenol blue	Merck 8122
16. Calcium chloride	Merck 2380
17. Calcium nitrate	Ajax 135
18. Carbenicillin disodium	Amresco J358
19. Cefotaxime, sodium salt	Amresco E868
20. Cetyldimethylethylammonium bromide (CTAB)	Sigma C5335
21. Chloroform	Merck 3445
22. Coomassie Brilliant blue R250	Bio-Rad 161 0400
23. Disodium 3-14-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.1^{3,7}] decan}-4yl) phenyl phosphate	Boehringer 1 655 884

24. Cupper sulfate, anhydrate	Sigma C1297
25. Cupric chloride, dihydrate	Sigma C6641
26. dATP	Boehringer 1277049
27. Diethyl pyrocarbonate	Sigma D5758
28. Disodium hydrogen phosphate	Sigma S0876
29. Dithiothreitol, DDT (100mM)	Promega P1171
30. EDTA, disodium salt	Sigma E5143
31. EDTA, ferrous-sodium salt	Sigma EDFS
32. EGTA, sodium salt	Sigma E3889
33. Ethanol (absolute)	Merck 100986
34. Ethidium bromide	Sigma E7637
35. Formaldehyde (37%)	Sigma F8775
36. Formamide	Boehringer 1814320
37. Genetamicin sulfate	Sigma G3632
38. Glacial acetic acid	Sigma A4508
39. Glycine	Sigma G7403
40. Hydrochloric acid (36%)	Ajax 1364
41. Imidazole	Sigma I2399
42. Indole-3-butyric acid	Sigma I1875
43. Iso-amylalcohol	Merck 100979
44. Isopropanol	Labscan C2519
45. Isopropyl b-D-thiogalactopyanside (IPTG)	Boehringer 1411446
46. Kanamycin, monosulfate	Sigma K4000
47. Lithium chloride	Sigma L8895
48. Luria Bertani broth, Miller	Difco 0446-17-3
49. Maleic acid	Sigma M0375

50. Magnesium chloride	Sigma M9272
51. Magnesium sulphate	Ajax 302
52. Mannitol	Ajax 530
53. β-mercaptoethanol	Sigma M6250
54. MES	Sigma 3023
55. Methanol	Merck 6007
56. Metro-mix soil	Hummert 10-0325
57. 4-Morpholineethanesulfonic acid	Boehringer 223794
58. MOPS	Sigma M8899
59. Murashige & Shoog salt mixture	GibcoBRL 11117-017
60. Myoinositol	Sigma I5125
61. N-lauroylsarcosine	Sigma L5125
62. Nicotine acid	Sigma N4126
63. Phenol-chloroform-isoamylalcohol (25:24:1)	Amersco 883
64. PIPES	B/M 239496
65. Polyvinylpyrrolidone	Sigma PVP-40T
66. Potassium acetate	Sigma P1147
67. Potassium hydroxide	Merck 5033
68. Potassium nitrate	Sigma P8394
69. Potassium phosphate, monobasic	Sigma P5379
70. Pyridoxine-HCl	Sigma P9755
71. Rifampicin	Sigma R3501
72. SDS	Bio-Rad 161-0302
73. Silwet-77	Lehle seeds
74. Sodium acetate, anhydrous	Sigma S2889
75. Sodium chloride	RDH 31434

76. Sodium citrate, trisodium salt	Sigma S4641
77. Sodium dihydrogen phosphate	RDH 10245
78. Sodium dodecyl sulfate	B/M 1028693
79. Sodium hydroxide	Merck 6498
80. Sodium molybdate	RDH 31439
81. Sucrose	Sigma S1888
82. Tris (hydroxyethyl) aminoethane	Ameresco 0826
83. Tris/ HCl	Amresco 0826
84. Tween 20	Bio-Rad 170-6531
85. TEMED	Bio-Rad 161 0800
86. Triton X-100	Sigma T6878
87. Urea	Sigma U1250
88. Zinc sulfate, heptahydrate	Sigma Z4750

Appendix III – Commercial Kits:

1. ABI prism dRhodamine terminator cycle sequencing ready reaction kit
   Peckin-Elmer 402078
2. Aurora™ western blot chemiluminescent detection system
   ICN 92626
3. Bio-rad Prep-A-Gene DNA Purification kit Bio-Rad 732 6011
4. Bio-Rad protein assay Bio-Rad 500-0006
5. Clone checker system GibcoBRL 11666-013
6. DIG RNA labeling kit Boehringer 1 175 025
7. High pure PCR product purification kit Boehringer 1732 668
8. MabTrap G II system Pharmacia. Biotech.71-5003-43
9. Rabbit reticulocyte lysate system Promega L4960
10. Ribomix large scale RNA production system-T7
    Promega P1300
11. Ribomix large scale RNA production system-SP6
    Promega P1280
12. Talon metal affinity resins Clontech 8901-1
13. Transcend non-radioactive translation detection system (Chemiluminescent)
    Promega 5080
18. Wheat germ extract Promega L4330
19. Wizard plus minipreps DNA purification kit Promega A7510

Appendix IV - Equipments and facilities used:

1. Biological Safety Cabinet	Baker SG600E 59419
2. Centrifuge J2-MI	Beckman T373 with JA-14 rotor
3. Dot blot microfiltration apparatus	Bio-Rad 170-3938
4. Gel 1000UV Fluorescent Gel Doc	Bio-Rad 200015450
5. Genetic Analyzer ABI Prism 310	Perkin elmer 96030481
6. Gene Pulser Apparatus	Bio-Rad 165-2076
7. Growth chamber	Percival AR-32L 3859-05-971
8. GS Gene Linker UV Chamber	Bio-Rad 0392-92-0336
9. Microcooler II	Bockel Scientific 260010
10. Orbital shaker	Lab line 4628-1
11. Power supply MIDI MP-250	Life technologies 4801311
12. Programmable Thermal Controller	MJ Research PTC100 96VHB 200003879
13. Refrigerated Centrifuge 5810R	Eppendorf 03463
14. Solvent System Centrivap Unit	Labconco 79840-01
15. TransBlot SD System	Bio-Rad 170-3949
16. TELCO incubator	Cole-Parmer 39352-02

Appendix V - Buffer, solution, gel and medium formulation

Acrylamide/Bis (30:0.15)	Disolving 60g acrylamide and 0.3g Bis in 200ml ddH_2O
Acrylamide/Bis (30:1.5)	Disolving 30g acrylamide and 1.5g Bis in 100ml ddH_2O
Agarose gel (0.8%)	0.8% agarose, 1μg/ ml ethidium bromide in 1X TAE buffer
Arabidopsis fertilizer (10X)	50mM KNO_3, 25mM 1M KPO_4 (pH 5.5), 20mM $MgSO_4$, 20mM $Ca(NO_3)_2$, 0.5mM FeNaEDTA and 1% micronutrients. Fill up to 1 litre with H_2O
Arabidopsis micronutrients	70mM boric acid, 14mM $MnCl_2$, 5mM $CuSO_4$, 0.2mM $NaMoO_4$, 10mM NaCl, and 0.01mM $CuCl_2$. Fill up to 500ml with H_2O.
B5 vitamine (1000X)	1000mg myo-inositol, 100mg thiamine-HCl, 10mg nicotine acid, 10mg pyridoxine-HCl. Fill up to 10ml with H_2O
Bacterial cell lysis solution	20mM Tris-HCl, 100mM NaCl, 8M urea
Blocking buffer	Dilute blocking reagent stock solution 1:10 with maleic acid buffer
Blocking reagent stock solution (10%)	Add 10g blocking reagent to 100ml maleic acid buffer with several 30s heat pulses in the microwave
Bromophenol blue loading dye (6X)	0.25% bromophenol blue in 30% glycerol
Calcium chloride solution	60mM $CaCl_2$, 15% glycerol and 10mM PIPES, pH 7.0, sterilized by autoclave
Cocultivation medium	4.3g/ L MS salt, 10mg/ L BA, 0.2mg/ L IBA, 0.3% sucrose, 1X B5 vitamin, 100μM AS, 7.6g/ L Bacto-agar
Cold washing solution	2x SSC, 0.1% SDS
Coomassie destaining solution	100ml absolute methanol, 25ml glacial acetic acid, add ddH_2O to 400ml final volume
Coomassie staining solution	0.5g Coomassie brilliant blue G-250, 5ml absolute methanol and 495 ml destaining solution

CTAB extraction buffer	0.1M Tris-HCl (pH8), 1.4M NaCl, 0.1M EDTA (pH8), 2% (w/v) CTAB, 1% (w/v) Polyvenylpyrolidone and 0.2% β-mercaptoethanol
CTAB washing buffer	76% EtOH with 0.01M NH_4OAc
Denaturation buffer	1.5M NaCl, 0.5M NaOH
DEPC-treated H2O	Dissolve DEPC to 1% in ultrapure H_2O and keep overnight Autoclave to remove residual DEPC.
Depurination solution	0.25M HCl
Detection buffer	100mM Tris-HCl, pH 9.5 and 100mM NaCl
Differentiation medium	4.3g/ L MS salt, 10mg/ L BA, 0.2mg/ L IBA, 0.3% sucrose, 1X B5 vitamin, 7.6g/ L Bacto-agar, 500mg/ L Carbenicillin or Cefotaxime
Explant preparatory medium	4.3g/ L MS salt, 10mg/ L BA, 0.2mg/ L IBA, 0.3% sucrose, 1X B5 vitamin, 100μM AS, 0.35M mannitol
Explant washing medium	4.3g/ L MS salt, 10mg/ L BA, 0.2mg/ L IBA, 0.3% sucrose, 1X B5 vitamin, 500mg/ L Carbenicillin or Cefotaxime
Germinating medium	4.3g/ L Murashige & Shoog salt mixture, 3% sucrose, 0.05% MES, pH 5.7
Glycine running buffer (SDS-PAGE gel running buffer)	0.05M Tris base, 0.38M glycine, 0.1% SDS, 2mM EDTA-Na
GUS staining solution	62mM Na_2HPO_4, 34M NaH_2PO_4, 0.5M $K_3[Fe(CN)_6]$, 0.5M $K_4[Fe(CN)_6]$, 1mM EDTA-Na, 1μg/ ml X-gluc
High SDS concentration hybridization buffer (northern blot)	7% SDS, 50% formamide, 5x SSC, 2% blocking reagent, 50mM sodium-phosphate, pH 7.0 and 0.1% N-lauroylsarcosine
His-tag protein elution buffer	20mM Tris-HCl, 100mM NaCl, 8M urea and 100mM Imidazole
Hot washing solution	0.5x SSC, 0.1% SDS
Infiltration medium	2.2g MS salts, 1x B5 vitamins, 50g sucrose, 0.5g MES and 200μl Silwet$_{TM}$

	L-77, 0.044μM benzylaminopurine. Adjust pH to 5.7 with KOH and fill up to 1 litre solution. Autoclave.
LB broth	25g/ L LB powder, autoclave
LB agar plate	25g/ L LB powder and 15g/ L bacto-agar, autoclave
Maleic acid buffer	0.1M maleic acid, 0.15M NaCl, pH 7.5. Adjust pH with concentrated NaOH; autoclave.
MOPS (10X)	200mM MOPS, 50mM sodium acetate, 10mM EDTA, pH 7.0. Make up in sterile H_2O. After autoclaving, the solution will turn yellow
MS plate	4.3g/ L Murashige & Shoog salt mixture, 3% sucrose, 0.05% MES, pH 5.7, 0.9% bacto-agar
Neutralization solution	0.5M Tris-HCl, 0.5M Tris-HCl, pH 7.5
N-lauroylsarcosine	10% (w/v) in sterile H_2O filtered through a 0.2μm membrane
Phosphate buffer saline (for GH transgenic plant protein extraction)	0.078M Na_2HPO_4, 0.122M NaH_2PO_4, 5mM ascorbic acid, 2% (w/v) PVP, 1mM β-mercaptoethanol and 0.1% Triton X-100, pH7
Phosphate buffer saline (for LCMV transgenic plant protein extraction)	0.58M Na_2HPO_4, 0.17M $NaH_2PO_4H_2O$, 0.68M NaCl, 2% (w/v) PVP and 2% (v/v) β-mercaptoethanol
Phosphate Buffer saline (PBS) (for blocking membrane during detection of *in vitro* transcripted and translated protein)	0.58M Na_2HPO_4, 0.17M $NaH_2PO_4H_2O$ and 0.68M NaCl
Protein transfer buffer	20mM Tris, 20mM glycine and 20% methanol
Resuspension medium	4.3g/ L MS salt, 10mg/ L BA, 0.2mg/ L IBA, 0.3% sucrose, 1X B5 vitamin, 100μM Acetosyringone
RNA extraction buffer	200mM Tris base, 400mM KCl, 200mM Sucrose, 35mM $MgCl_2 \cdot 6H_2O$, 25mM EGTA, pH 9
RNA loading buffer	250μl formamide, 83μl formaldehyde

	37% (w/v), 50µl 10x MOPS buffer, 0.01% (w/v) bromophenol blue, 50µl glycerol. Fill up to 500µl with DEPC-treated H_2O.
SDS	10% (w/v) in sterile H_2O filtered through a 0.2µm membrane
SDS polyacrylamide separation gel (10%)	4.18ml Acrylamide/Bis (30: 0.15), 1.25ml 3.5M Tris/HCl (pH 8.8), 0.125ml 0.2M EDTA, 0.125ml 10% SDS, 0.12ml 7.5% ammonium persulfate, 0.015ml TEMED, add sterile H_2O to 12.515ml final volume
SDS polyacrylamide separation gel (15%)	6.25ml Acrylamide/Bis (30: 1.5), 1.25ml 3.5M Tris/HCl (pH 8.8), 0.125ml 0.2M EDTA, 0.125ml 10% SDS, 0.12ml 7.5% ammonium persulfate, 0.015ml TEMED, add sterile H_2O to 12.515ml final volume
SDS sample buffer (for SDS-PAGE gel electrophoresis)	20% SDS, 25µg/ ml, bromophenol blue, 0.5M Tris-HCl (pH6.8), 5% β-mercaptoehtanol
SOC	2% bacto-tryptone, 0.5% bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose
Sodium acetate	3M NaOAc, pH 5.2 3M NaOAc, pH 5.6
Sodium phosphate	1M NaH_2PO_4, 1M Na_2HPO_4, pH7
SSC (20X)	3M NaCl, 300mM sodium citrate, pH 7.0
SDS polyacrylamide stacking gel	0.5ml Acrylamide/Bis (30: 0.15), 0.5ml 1.25M Tris/HCl (pH 8.8), 0.05ml 0.2M EDTA, 0.05ml 10% SDS, 0.04ml 7.5% ammonium persulfate, 0.006ml TEMED, add sterile H_2O to 5ml final volume
Stimulating medium	4.3g/ L MS salt, 0.5mg/ L BA, 0.05mg/ L IBA, 0.3% sucrose, 1X B5 vitamin, 7.6g/ L Bacto-agar, 500mg/ L Carbenicillin or Cefotaxime
TAE buffer (1X)	4.84g/ L Tris base, 0.1142% acetic acid, 0.744g/ L EDTA disodium salt
Tris-acetate (1X)	0.04M Tris acetate and 0.001M EDTA

Tris buffered saline (for *in vitro* translated protein detection)	20mM Tris-HCl (pH7.5) and 150mM NaCl
Tris buffered saline with Tween 20 (for *in vitro* translated protein detection)	20mM Tris-HCl (pH7.5), 150mM NaCl and 0.5% Tween 20
Wash buffer (for washing membrane during detection of in vitro transcripted and translated protein)	0.58M Na_2HPO_4, 0.17M NaH_2PO_4 H_2O, 0.68M NaCl and 0.1% (v/v) Tween-20
YEP meidum	10g tryptone, 10g yeast extract and 5g NaCl. Fill up with 1 litre with H_2O